New Enzymatic Assay for Serum Urea Nitrogen Using Urea Amidolyase

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We established an enzymatic assay for measurement of serum urea nitrogen using urea amidolyase (EC 3.5.1.45) from yeast species. The method is based on hydrolysis of urea by the enzyme. In this assay, we eliminated endogenous ammonium ion by use of glutamate dehydrogenase (EC 1.4.1.4). Then in the presence of urea amidolyase, ATP, bicarbonate, magnesium, and potassium ions, ammonium ion was produced proportionally to urea concentration in serum. The concentration of ammonium ion formed was determined by adding GLDH to produce NADP⁺ in the presence of 2-oxoglutarate and NADPH. We then monitored the change of absorbance at 340 nm. The inhibitory effect of calcium ion on this assay was eliminated by adding glyco-

letherdiamine-N, N, N', N'-tetraacetic acid to the reaction system. The within-assay coefficient of variations (CVs) of the present method were 1.80–3.76% (n = 10) at 2.8–19.0 mmol/L, respectively. The day-to-day CVs were 2.23–4.59%. Analytical recovery was 92–115%. The presence of ascorbic acid, bilirubin, hemoglobin, lipemic material, ammonium ion, or calcium ion did not affect this assay system. The correlation between values obtained with the present method (y) and those by another enzymatic method (x) was 0.997 ($y = 1.02x - 0.10$ mmol/L, $Sy/x = 0.841$, n = 100), with a mean difference of -0.18 ± 0.86 mmol/L [(values by reference method – that of present method) $\pm$ SD] using the Bland-Altman technique. J. Clin. Lab. Anal. 17:52–56, 2003. © 2003 Wiley-Liss, Inc.

Key words: SUN; enzymatic assay; urea amidolyase; automatic analysis

INTRODUCTION

Urea is a degradation product of protein metabolism, which is synthesized predominantly by the urea cycle in the liver with ammonia and carbon dioxide. The excreted urea is filtered by the glomerulus, and part of this is reabsorbed from the uriniferous tube. Urea has been measured as urea nitrogen (urea N), and urea N constitutes about 45% of the total nonprotein nitrogen (1). Blood or urinary urea N is an important indicator of liver and/or kidney function, and the concentration of urea N is widely determined by the use of urease (EC 3.5.1.5). Methods that use urease (which catalyzes the hydrolysis of urea to ammonia and carbon dioxide) include 1) the indophenol blue method (2), in which the ammonia produced from urea is determined by Berthelot reaction, using sodium nitroprusside as the catalyst; 2) the enzyme coupling method, in which glutamate dehydrogenase (EC 1.4.1.4, GLDH) is used (3); and 3) the Vitros method (4), in which formed ammonia is reacted with a protonated merocyanine dye as an indicator.

Urea amidolyase (EC 3.5.1.45) is a multifunctional enzyme that requires divalent and monovalent ions, such as magnesium, potassium, and ammonium ions, for its activation, and calcium ion for its inhibition. This enzyme is unique in nature for these reasons. We examined the possibility of using urea amidolyase for classifying some ions as activators or inhibitors, and using urea as a substrate in serum as a model of the enzymatic method. Previous studies (5–7) have shown that this enzyme is useful for determining ions, such as potassium, magnesium, and calcium, in serum. In the present work we report a new enzymatic assay for serum urea N (SUN). This assay uses urea amidolyase, which is a multifunctional enzyme that catalyzes the ATP-dependent cleavage of urea. This enzyme catalyzes two

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reactions in association with urea cleavage: 1) carboxylation of urea with urea carboxylase (EC 6.3.4.6), and 2) hydrolysis of allophanate with allophanate hydrolase (EC 3.5.1.54) (8,9). We applied this property to develop an automated assay for SUN. In this work we evaluate the method and examine its correlation with another enzymatic method for SUN concentrations.

MATERIALS AND METHODS

Apparatus

Enzymatic measurement of SUN was performed with the Hitachi model 7070 (also known as the Hitachi 911 in the U.S. and Europe) automatic analyzer (Hitachi Co. Ltd., Tokyo, Japan). For comparison, the conventional method was performed with the Hitachi 7450 (also known as the Hitachi 747 in the U.S. and Europe) automatic analyzer (Hitachi Co. Ltd.).

Reagents

Analytical-grade potassium chloride, ammonium chloride, calcium acetate, magnesium acetate, sodium bicarbonate, urea, 2-oxoglutaric acid, and hydrochloric acid were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The NADPH (Oriental Yeast Co., Ltd., Osaka, Japan), lipemic turbidity, hemoglobin, and bilirubin (International Reagent Co., Ltd., Kobe, Japan) were also of analytical grade. This was obtained from Sigma Aldrich Co., Ltd. (St. Louis, MO), and the glycoetherdiamine-N, N, N', N'-tetraacetic acid (GEDTA) was from Dojindo Laboratories (Kumamoto, Japan). GLDH and urea amidolyase (URL-311) were purchased from Toyobo Co., Ltd. (Osaka, Japan).

Preparation of reagents

Tris-HCl buffer (200 mmol/L; pH 8.0 at 37°C), containing 1.0 μ mol/L GEDTA, 3.0 mmol/L magnesium acetate, and 19.5 mmol/L potassium chloride was used as the buffer solution. The first reagent consisted of ATP 1.9 mmol/L, sodium bicarbonate 20.7 mmol/L, 2-oxoglutaric acid 9.7 mmol/L, NADPH 0.29 mmol/L, and GLDH 20 kU/L in the buffer solution. The second reagent contained sodium bicarbonate 20.7 mmol/L and urea amidolyase 644 U/L in the buffer solution.

The calibrators were physiological saline with and without 18.0 mmol/L urea N. The conversion factor for mmol/L to mg/dL is Urea N mmol/L = Urea N mg/dL $\times$ 0.3569.

Assay Procedure

The assay was performed with a two-point, fixed-time rate assay at 37°C with the Hitachi 7070 automatic analyzer. Either the calibrator solution or the serum (10 μ L) was mixed with 350 μ L of diluent, and 4 μ L of dilution sample were mixed with 250 μ L of the first reagent and kept for 1 min at 37°C. After the addition of 50 μ L of the second reagent, the absorbance was monitored from 4–9 min, and the rate of reaction catalyzed by urea amidolyase was measured by the decrease of absorbance at 340 nm for 5 min at 20-sec intervals, using the absorbance at 405 nm as a reference.

Comparison Method

We also determined the SUN concentration by another enzymatic method reagent kit (Selotec, Hokkaido, Japan). This enzymatic method is based on hydrolysis of urea by urease.

Specimens

Serum specimens were collected from 100 patients with various diseases in Osaka University Hospital. The patients were informed that samples of their blood were being used for research, and that their privacy would be protected.

Statistics

Correlation between the SUN concentration values obtained by the present method and another enzymatic method was determined by linear regression analysis. We also statistically compared the present method with another enzymatic method by the Bland-Altman method (10), and the results are presented as the mean $\pm$ SD.

RESULTS

Studies for Optimum Assay Condition

We examined the effects of pH on urea amidolyase activity in Tris-HCl buffer solutions at pH 7.5–8.3. We observed maximal activity at pH 7.5–8.0, and under these conditions the K_m value was 0.44 mmol/L for urea.

We also attempted to determine the optimal concentrations of ATP, bicarbonate ion, potassium ion, magnesium ion, and urea amidolyase. The K_m values were obtained from Lineweaver-Burk plots for this enzyme, 0.15 mmol/L for ATP, 2.04 mmol/L for bicarbonate ion, and 1.92 mmol/L for potassium. The final concentrations of substrate and coenzyme were >10 -fold for each K_m value. A maximal reaction was observed when the concentration of magnesium ion was ~ 3.0 mmol/L; therefore, the assay was performed under

Fig. 1. Typical time course for a bicarbonate ion assay. The time course of the overall reaction from 0 to 10 min is shown, using a standard solution of urea N (18.0 mmol/L) containing 5.0 mmol/L ammonium ion.

that condition. The optimal activity of urea amidolyase was obtained with a final concentration of 0.106 U/L; at this concentration the time course of the reaction showed the most quantitative and linear curve.

To determine GLDH activity, we obtained a reaction end point within 1 min with concentrations >20 kU/L; we employed this unit value for the final concentration of GLDH.

Figure 1 shows a typical time course for the first reaction (elimination of endogenous ammonium ion) and the second reaction (measurement of urea N concentration). The first reaction terminated within 1 min and then absorbance decreased linearly with time. Absorbance for the second reaction was read from 4–9 min after addition of the second reagent.

Performance of the SUN Assay

The calibration curve for urea N standard solution was linear up to at least 57.1 mmol/L, as shown in Fig. 2.

We estimated the detection limit of the present method by assaying the zero calibrator 10 times, and the result (mean $\pm$ SD) was 0.41 ± 0.33 mmol/L. The functional sensitivity, determined by serial dilution of 10 mmol/L urea N solution with zero calibrator, was 1–1 mmol/L (mean coefficient variation (CV)), we decided the functional detection limit of urea N to 1.1 mmol/L to obtain result with good precision in low level of urea N assay.

Fig. 2. Standard curve for the enzymatic assay of urea N. The regression equation between the reaction rate (Δ absorbance at 340/405 nm/5 min) (y) and the urea N concentration (mmol/L) (x) was $y = 0.0016x - 0.0003$ ($r = 0.99$).

TABLE 1. Precision of SUN assay

Assay	Sample		
	1	2	3
Within-assay (n = 10)			
Mean (mmol/L)	2.8	6.9	19.0
SD (mmol/L)	0.11	0.24	0.34
CV (%)	3.76	3.53	1.80
Day-to-day assay (n = 10)			
Mean (mmol/L)	2.3	5.9	17.0
SD (mmol/L)	0.11	0.21	0.38
CV (%)	4.59	3.54	2.23

The within-assay and day-to-day reproducibility for three different human pooled sera was tested. As shown in Table 1, within-assay CVs ranged from 1.89% to 3.76%. Day-to-day CVs were 2.23–4.59%.

The analytical recoveries for urea N (1.0, 2.0, 3.0, and 5.0 mmol/L) added to three different human pooled sera were 92–115%.

We found no interference by the addition to pooled human sera with 6.1 mmol/L and 10.0 mmol/L SUN of 100 mmol/L Na^+ , 5.0 mmol/L NH_4^+ , 5.0 mmol/L Ca^{2+} , 224 mg/L conjugated bilirubin, 186 mg/L unconjugated bilirubin, 4–8 g/L hemoglobin, and 1670 formazine turbidity units of lipemic material.

We examined the correlation between the SUN values obtained by the present method (y) and another enzymatic method (x). A total of 100 samples were

Fig. 3. Correlation of SUN concentration with 100 samples from patients. **A:** The regression line analysis of the present method (y) vs. another enzymatic method (x) was $0.997 (y = 1.02x - 0.103 \text{ mmol/L}, S_{y/x} = 0.841)$. **B:** The difference between the SUN values of the present method and another enzymatic method are shown in a Bland-Altman plot. The mean value of the two methods is plotted against the difference between the two values. The mean difference ($\pm$ SD) between the two methods was -0.18 ± 0.86 . The mean difference (solid line) and SD ($\pm$ SD) of the mean difference (dotted line) is shown parallel to the x-axis.

measured, resulting in mean values of SUN concentration of 13.2 mmol/L for the present method, and 13.1 mmol/L for the other method. The relationship and regression line between the present method and the other method is shown in Fig. 3A. The degree of agreement between the methods was assessed using the Bland-Altman graphical technique, as shown in Fig. 3B. The mean bias was -0.18 (SD = 0.86, with a 95% confidence interval of -0.35 to -0.01) for values of the other enzymatic method minus those of the present method.

DISCUSSION

Urea amidolyase catalyzes the cleavage of urea with the formation of carbon dioxide and ammonia. This reaction is accompanied by the cleavage of ATP, with the liberation of equimolar amounts of ADP and inorganic phosphate, which is strictly dependent for activity on divalent (magnesium or manganese) and monovalent (potassium or ammonium) cations, and requires catalytic amounts of bicarbonate ion. However, the calcium ion is a very strong inhibitor of this enzyme. We believe that the calcium ion affects the enzyme-ATP-Mg complex by the time of urea carboxylation. The pattern of inhibition between calcium and magnesium ions was noncompetitive at pH 8.0, and the K_i value for the calcium ion was $6.8 \times 10^{-5} \text{ mol/L}$. Furthermore, the inhibition between calcium and ATP was noncom-

petitive at pH 8.0. EDTA and GEDTA are widely used as chelating agents (11,12); however, EDTA cannot be used in the present assay because the affinity of EDTA for the magnesium ion is very strong, and magnesium ions are required for activation of urea amidolyase. The affinity of GEDTA for the calcium ion is stronger than that for the magnesium ion; therefore, we used GEDTA as a chelating agent to remove the calcium ion. Because the serum calcium ion concentration ranged from 2.0 to 5.0 mmol/L, the serum sample was prediluted 36-fold for the automatic analyzer. Furthermore, the prediluted sample was diluted 76-fold with the reagents in our assay, and the final concentration of calcium ion in the assay medium was $\sim 0.731\text{--}0.914 \mu\text{mol/L}$. We therefore added $0.1 \mu\text{mol/L}$ GEDTA to the reaction system. The present enzymatic method is precise, offers reasonable analytical recovery without possible interference from a number of materials, and shows good correlation with the conventional enzymatic method. The assay takes only 10 min to perform, and can be performed by a general random-access biochemical analyzer.

REFERENCES

1. Giorgio JD. Non-protein nitrogenous constituents. In: Henry RJ, Cannon DC, Winkelman JW, editors. *Clinical chemistry: principles and techniques*. 2nd ed. London: Harper&Row; 1974. p 504–557.
2. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem* 1962;8:130–132.

3. Hallett CJ, Cook JGH. Reduced nicotinamide adenine dinucleotide-coupled reaction for emergency blood urea estimation. *Clin Chim Acta* 1971;35:33–37.
4. Spayd RW, Bruschi B, Burdick BA, et al. Multilayer film elements for clinical analysis: applications to representative chemical determinations. *Clin Chem* 1978;24:1343–1350.
5. Kimura S, Iyama S, Yamaguchi Y, et al. New enzymatic assay for calcium in serum. *Clin Chem* 1996;42:1202–1205.
6. Kimura S, Iyama S, Yamaguchi Y, et al. New enzymatic assay with urea amidolyase for determining potassium in serum. *Ann Clin Biochem* 1997;34:384–388.
7. Kimura S, Iyama S, Yamaguchi Y, et al. Enzymatic assay for magnesium in serum. *Ann Clin Biochem* 2000;37:82–84.
8. Roon RJ, Levenberg B. Urea amidolyase. I. Properties of enzyme from *candida utilis*. *J Biol Chem* 1972;247:4107–4113.
9. Roon RJ, Hampshire J, Levenberg B. Urea amidolyase: the involvement of biotin in urea cleavage. *J Biol Chem* 1972;247:7539–7545.
10. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–310.
11. Burg RA, Conaghan HF. Chelometric determination of calcium and magnesium in minerals. *Chem Analyst* 1960;49:100–102.
12. Austin JF, Klett CA. EGTA titration of calcium in algae sample high in magnesium and phosphate. *Chem Analyst* 1966;55:11–13.