Improved Kinetic Rate Assay of Urinary N-Acetyl-β-D-glucosaminidase with 2-Chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide as Substrate

Junko Makie,1 Emiko Saito,1 Miyako Obuchi,1 Masashi Kanayama,2 Klyomi Harakawa,2,3 and Kenji Yoshida3

We have improved the kinetic rate assay method for determining N-acetyl-β-D-glucosaminidase (EC 3.2.1.30; NAG) activity in urine with use of the synthetic substrate, 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG), reported previously (Clin Chem 1988;34:2140-2). To increase the solubility of this substrate, we used crown ether (15-crown-5-ether) and ethylene glycol. In addition, we used for the standard solution NAG from human placenta, with specificity corresponding to that of human urine, so that values obtained with the CNP-NAG method and a p-nitrophenyl-NAG method ("MEI Assay NAG") correlated almost completely (r = 0.995, n = 29). Reference values for urinary NAG activity determined by the CNP-NAG method were established for untimed urine specimens from 674 healthy volunteers. The normal reference interval (mean ± 2 SD) for NAG: 1.6-15.0 (mean 4.9) U per gram of creatinine.

Additional Keyphrases: centrifugal analyzer · reference interval · diabetes, renal disease · Km values

Various types of synthetic substrates have been reported for use in determinations of urinary N-acetyl-β-D-glucosaminidase (EC 3.2.1.30; NAG) (I-3,6). To determine urinary NAG activity by a kinetic rate assay method, we used 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG), a new synthetic substrate, as already described (I). This method allowed good reproducibility in accordance with the reaction rate theory; required no specimen blank; was unaffected by endogenous components of urine such as glucose, ascorbic acid, and bilirubin; and could be adapted to various types of automated analyzers. However, the substrate is sparingly soluble (≤2.0 mmol/L) in water, and the reagent mixture is slightly unstable after dissolution. Moreover, we found differences in specificity between NAG from bovine kidney and NAG from human tissue when we determined NAG activity by comparison with standard solutions instead of basing results on the molar absorptivity of CNP.

Here we report several improvements to this method.

Principle

CNP-NAG, hydrolyzed by NAG at 37 °C and pH 5.7, liberates the chromogen, CNP. NAG activity is determined by calculating the rate of CNP formation, in terms of the change in absorbance per minute at 400 nm. Changing the reaction pH from 4.7, as in the original method (I), to 5.7, improved our results.

Materials and Methods

Reagents: We used CNP-NAG (Meiji Seika Kaisha, Ltd., Tokyo, Japan), crown ether (15-crown-5; Nippon Soda Co., Ltd., Tokyo, Japan), ethylene glycol (Junsei Chemical Co., Ltd., Osaka, Japan), and citric acid and citric acid sodium salt (both special grade; Koso Chemical Co., Ltd., Tokyo, Japan). All other reagents used were of special grade or for biochemical use. For comparison, we used NAG assay kits with p-nitrophenyl-N-acetyl-β-D-glucosaminide (PNP-NAG) and m-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide (MCP-NAG) as substrates, obtained from Meiji Seika Kaisha and Shionogi & Co., Ltd., Osaka, Japan, respectively.

Urine samples: Pooled untimed urine specimens were from unselected inpatients and outpatients of this hospital. Urine specimens from ostensibly normal healthy subjects were used to establish the reference values for healthy persons.

Composition of reagents: Reagent I was citrate buffer (0.2 mol/L, pH 5.6). Reagent II consisted of 22 mg of CNP-NAG dissolved in 3 mL of an equimolar mixture of crown ether and ethylene glycol, then mixed with 2 mL of isotonic saline. Final concentrations of each reagent used in the Cobas Bio centrifugal analyzer (Hoffmann-La Roche, Basel, Switzerland) were, per liter: CNP-NAG, 2.1 mmol; pH 5.7 citrate buffer, 145 mmol; 15-crown-5 ether, 54.6 g; ethylene glycol, 54.6 g; and NaCl, 0.7 g. NAG activities were measured at pH 5.7.

Procedures

Determining NAG activity with the Cobas Bio: To 20 μL of urine sample in the centrifugal analyzer, add 200 μL of reagent I (buffer) and, 10 s later, 50 μL of reagent II (substrate solution). For the rate assay, measure the absorbance at 400 nm 10 s later and then for 3 min.

The assay was carried out at 37 °C, and NAG activity was calculated on the basis of values for the standard solution derived from human placenta.

Determining activity values of standard solutions of NAG derived from human placenta: To determine the activity of this standard solution by the PNP-NAG method, we established NAG activity values according to the molar absorptivity of PNP released in the PNP-NAG method ("MEI

1 Central Clinical Laboratory, and 2 Department of Internal Medicine, Yokosuka Kyosai Hospital, 1-18 Yonegahama-dori, Yokosuka, Kanagawa, 238 Japan.
3 Nonstandard abbreviations: NAG, N-acetyl-β-D-glucosaminidase; CNP-NAG, 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminidase; PNP-NAG, p-nitrophenyl-N-acetyl-β-D-glucosaminidase; and MCP-NAG, m-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminidase.
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ASSAY NAG, Meiji Seika Kaisha Ltd.), p-Nitrophenyl (PNP) solution for determining the molar absorptivity was added to citrate buffer (50 mmol/L, pH 4.4) and the pH was adjusted to 10.7 with 0.25 mol/L Na₂CO₃. The molar absorptivity of PNP in the pH-adjusted buffer, measured with a Shimadzu UV 240 spectrophotometer at 405 nm, was 18.04 L·cm⁻¹·mol⁻¹.

Results

Analytical Variables

CNP-NAG solubility was increased to 27 mmol/L in the equimolar mixture of crown ether and ethylene glycol, and the dissolution rate was enhanced. In this solvent the reagents were stable for as long as 10 days when stored at 4 °C, although the absorbance of the reagents increased slightly with the passage of time (Figure 1).

Using various samples, we evaluated the relation between changes of CNP-NAG concentration (up to 10 mmol/L) and NAG activity. Figure 2 depicts the Lineweaver–Burk plots. The K_m values determined with this method showed similar reactivity for each sample type.

The specificity of NAG from human placenta and that of NAG from bovine kidney were compared (Table 1). When the NAG activities from human placenta determined by the PNP-NAG method, the MCP-NAG method, and the present method were all set at 100, so as to express relative activities (in percent), NAG activities in bovine kidney varied among the three methods, but NAG activities in human urine specimens were in fair agreement by each method.

Figure 3 shows the relation between results by the PNP-NAG method and the CNP-NAG method, obtained with (a) NAG from bovine kidney and (b) NAG from human placenta used in the standard solution. For both methods the correlation was good (r = 0.995). Therefore, the use of NAG from human placenta in the standard solution was valid.

There was good correlation (r = 0.996) between results by the original CNP-NAG method (x), in which activity values were measured by using the molar absorptivity of CNP, and those of the present method (y), based on use of standard solution: y = 0.96x – 5.8 U/L. The correlation between the MCP-NAG method (x') and the present method was also good: y = 1.91x – 0.03 U/L (r = 0.996, n = 29), with the present method showing about double the activity values of the MCP-NAG method.

NAG Activity in Control Subjects and Patients

To determine the distribution of urinary NAG activity in

![Graph](image-url)

Fig. 1. Stability of NAG assay reagents stored at 4 °C: (©) urine, (●) bovine NAG, (○-○) blank

![Graph](image-url)

Fig. 2. K_m values of NAG for CNP-NAG by Lineweaver–Burk plots: human placenta NAG (©), K_m = 0.125 × 10⁻⁴ mol/L; human urine (○), K_m = 0.167 × 10⁻⁴ mol/L; bovine kidney NAG (△), K_m = 0.25 × 10⁻⁴ mol/L

![Graph](image-url)

Fig. 3. Correlation between results by the PNP-NAG method and the present CNP-NAG method: (a) y = 0.68x – 0.7 U/L, r = 0.995, n = 29; (b) y = 1.04x – 1.0 U/L, r = 0.995, n = 29

![Table](image-url)

Table 1. Comparison of NAG Specificity in Modified Kinetic Assay of NAG

<table>
<thead>
<tr>
<th>Method</th>
<th>Human placenta</th>
<th>Bovine kidney</th>
<th>Human urine</th>
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<tbody>
<tr>
<td>PNP-NAG</td>
<td>100</td>
<td>47</td>
<td>74</td>
</tr>
<tr>
<td>CNP-NAG</td>
<td>100</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td>MCP-NAG</td>
<td>100</td>
<td>93</td>
<td>75</td>
</tr>
</tbody>
</table>
NAG activity
(U/g creatinine)

<table>
<thead>
<tr>
<th>Value (U/L)</th>
<th>Males (n)</th>
<th>Females (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00-1.00</td>
<td>2-5</td>
<td></td>
</tr>
<tr>
<td>1.00-2.00</td>
<td>2-5</td>
<td></td>
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<tr>
<td>2.00-3.00</td>
<td>2-5</td>
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</tr>
<tr>
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<td>4.00-6.00</td>
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<tr>
<td>6.00-7.00</td>
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<td>8.00-10.00</td>
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<tr>
<td>10.00-12.00</td>
<td>2-5</td>
<td></td>
</tr>
<tr>
<td>12.00-15.00</td>
<td>2-5</td>
<td></td>
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</tbody>
</table>

Fig. 4. Reference values for healthy individuals: mean ± 2 SD = 1.6-15.0 (mean 4.9) U/g of creatinine (n = 674)

healthy persons, we used untimed urine specimens from 674 subjects, all apparently healthy. Their age ranges and sex are shown in Figure 4. The results showed a logarithmic normal distribution. The reference interval was 1.1-20.3 (mean 4.7) U/L, expressed as the mean ± 2 SD by the iterative truncation method, and 1.6-15.0 (mean 4.9) U per gram of creatinine when corrected for differences in excretion rates by use of data on the creatinine concentration.

Diabetes mellitus patients treated in this hospital, excluding those with diabetic nephropathy or other renal disease complications, were divided into 32 with poorly controlled diabetes (high values for blood-sugar and HbA1c) and 59 with clinically well-controlled diabetes. We measured urinary NAG activity (U/L) and NAG index (U per gram of creatinine) in both groups, using untimed urine specimens; the results are shown in Figure 5. Values for both NAG activity and NAG index were higher in the poorly controlled group than in the well-controlled group. In the poorly controlled group, 11 cases showed normal values for NAG activity and only one case showed a normal value for the NAG index.

Discussion

Our original method (1) requires no specimen blank, has a small lag time, and allows NAG activity determination by tracing the reaction rate for about 3 min. However, the problem with the original method is that a final substrate concentration >2.0 mmol/L cannot be obtained. We examined many types of solubility-enhancing surfactants, but none showed an effect exceeding that of Triton X-100, which we used in the original method. Solubility enhancement was also examined by using α-cyclodextrin, an inclusion compound, and crown ether. Of the two, crown ether was found capable of enhancing both the solubility of the substrate and its stability in solution when used in combination with ethylene glycol. In a 1:1 solution of crown ether and ethylene glycol, the maximum solubility of CNP-NAG in the method was 27 mmol/L, and a 16 mmol/L concentration was attained in 1–2 min. Moreover, the reagents after dissolution were far more stable than before, being usable for up to 10 days if stored at 4 °C.

This method allows evaluation of NAG activity by using the molar absorptivity of CNP at 37 °C and 400 nm. However, use of a standard solution was considered more desirable, because the molar absorptivity is apt to vary with changes in pH and reagent compositions, or instrumental differences in optical properties of analyzers. However, the NAG used in conventional NAG kits to prepare the standard solution was from bovine kidney. This has a different specificity from human urinary NAG and the range of standard solution concentrations is limited. When we used NAG from human placenta and compared it with urinary NAG, specificity agreement was confirmed, as shown in Table 1. Activity values obtained from molar absorptivity of PNP, when we used PNP-NAG method as the reference method, were indicated as NAG activity units in this method. Consequently, the regression formula for the correlation between results by the original CNP-NAG method and the present method was y = 0.96x - 5.8 U/L, a value slightly lower for the present method.

The clinical significance of NAG has been emphasized: it is useful both in screening and as a marker for the diagnosis and follow-up of the clinical course in various types of renal diseases (4-6). Also, it has been gaining attention recently for use in the early detection and observation of clinical course of diabetic nephropathy (7-9). The prognosis of diabetic nephropathy is variously unfavorable, and lesions of the renal tubules reportedly are related to the nephropathy in its early stages. NAG activity is determined in patients who are under long-term blood-glucose control, to aid in early diagnosis of diabetic nephropathy and in its follow-up.

Our results (Figure 5) reveal that many patients in the poorly controlled group showed remarkably high values for NAG activity compared with the well-controlled group and that the possibility of complication with renal disease is high in the poorly controlled group. Therefore, we consider measurement of NAG activity to be useful as a good index of renal dysfunction. Furthermore, NAG activity shows a broader range in urine from partially controlled diabetes;
the NAG index thus seems to have a high specificity.

Moreover, the clinical significance of NAG isoenzymes has also been examined and expectations of their future usefulness are good (7–10). Severini et al. (7) showed that the proportion of the B form in urine of diabetics with vascular changes was significantly higher than in control.

In conclusion, the present method is easily applicable to various types of automated analyzers and allows fast determination with good reproducibility. It should be useful in view of the prospect that NAG activity will be increasingly determined in the future.

References

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Hyponatremia in Patients Admitted to a Coronary Care Unit
John R. Evans,¹ John P. McIntosh,¹ H. John McIntosh,² and Peter E. G. Mitchell²

Calculation of osmolal gap in plasma and urine samples from patients after acute myocardial infarction was carried out to see whether changes in these variables support the concept of a "sick-cell" mechanism being responsible for the hyponatremia associated with acute myocardial infarction. Some supportive evidence was found on the first day after admission but, overall, the evidence was not convincing.

Additional Keyphrases: acute myocardial infarction - osmolal gap - urine

Systemic metabolic changes follow acute myocardial infarction including increased plasma concentrations of catecholamines, cortisol, renin, angiotensin II, and arginine vasopressin (AVP) (1–6).

Flear and Hilton (7) showed that hyponatremia is common in patients with confirmed AMI and that the degree of illness correlates roughly with the decrease in plasma sodium. The hyponatremia is transient, being greatest on day 3 or 4 after admission, then returning towards first-day plasma sodium concentrations by day 6 or 7. The average decrease in plasma sodium is about 5 mmol/L in the most severely affected group, with smaller changes in the less-affected patients. Flear and Hilton (7) thought this hyponatremia could be caused by a redistribution of body water and solutes, due to a widespread increase in cell-membrane permeability (i.e., the "sick-cell syndrome") or to a net gain of water caused by a stress-related sustained secretion of AVP.

We undertook this study to seek evidence for the involvement of the sick-cell syndrome mechanisms in the pathogenesis of hyponatremia in AMI, in particular by measurement of osmolal gaps in plasma and urine.

Patients and Methods

Patients

We studied 57 patients who were admitted with chest pain and suspected AMI; patients diagnosed as having other serious illnesses were not included. The patients studied received therapy as indicated clinically. There were no restrictions on oral fluid intake. Each patient was assessed clinically and placed into one of three groups according to the following criteria:

- Group 1 (24 patients)—no definite evidence of infarct, as assessed clinically, and without electrocardiograph changes or increased plasma concentrations of aspartate aminotransferase (AST) and a-hydroxybutyrate dehydrogenase (HBD) activities.
- Group 2 (18 patients)—definite evidence of infarct, with electrocardiograph changes and increases in plasma concentrations of AST and HBD activities, but without any of the additional criteria specified for Group 3.
- Group 3 (15 patients)—definite evidence of infarct, with electrocardiographic changes and increases in the concentrations of AST and HBD activities in plasma, plus any of the following specific criteria: HBD >1050 U/L (3 × upper reference limit), systolic blood pressure <100 mmHg, left or right ventricular failure, cardiogenic shock, worsening grade of failure, ventricular arrhythmias requiring therapy, or cardiac arrest.

³ Departments of ¹ Biochemical Medicine and ² Medicine, Strathclyde Hospital, Brenchin, Scotland, DD9 7QA, U.K.
⁴ Department of Biochemical Medicine, Ninewells Hospital, Dundee, Scotland, DD9 9SY, U.K.
⁵ Nonstandard abbreviations: AMI, acute myocardial infarction; AVP, arginine vasopressin; AST, aspartate aminotransferase (EC 2.6.1.1); and HBD, a-hydroxybutyrate dehydrogenase (EC 1.1.1.30).

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