Simple, Rapid Spectrophotometry of Urinary N-Acetyl-β-D-glucosaminidase, with Use of a New Chromogenic Substrate

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We have developed a new spectrophotometric assay for urinary N-acetyl-β-D-glucosaminidase (NAGase) with use of sodio m-cresol sulfonphthaleinyl N-acetyl-β-D-glucosaminide (MCP-NAG). MCP-NAG was synthesized from acetochlorogluco- somine and m-cresol sulfonphthalein (MCP) in four steps. MCP-NAG reacts well with NAGase (Km = 0.41 mmol/L) and is highly water soluble. The absorption maximum and molar absorptivity of the aglycone MCP are 580 nm and 40,670, respectively. Spectral overlap of interfering substances at 580 nm is almost negligible, so that the urine blank can be omitted from the assay procedure. The high molar absorptivity of MCP gives sufficient analytical sensitivity at a reaction time of 15 min. The correlation between the MCP-NAG method (y) and the fluorimetric method (x) involving 4-methylumbelliferyl N-acetyl-β-D-glucosaminide is represented by the equation y = 0.995x – 0.669 (r = 0.991). Thus, the present method provides practical advantages over conventional methods, for use in the routine laboratory.

**Materials and Methods**

**Apparatus.** Ultraviolet and visible spectra were measured with a Model 124 spectrophotometer (Hitachi Ltd., Ibaragi, Japan). For routine assay, absorbances were measured with a Model CL-720 micro-flow spectrophotometer (Shimadzu Co., Kyoto, Japan). A Model 204 spectrofluorimeter (Hitachi Ltd.) and a Model HM-18ET pH meter (Toa Electronics, Tokyo, Japan) were used.

**Reagents.** NAGase (bovine kidney) was purchased from Boehringer, Mannheim, F.R.G.; β-glucosidase (from sweet almond) from Miles Laboratories, Elkhart, IN 46515; β-galactosidase (from jack bean) from Seikagaku Kogyo, Tokyo, Japan; β-glucuronidase (from Escherichia coli) from Sigma Chemicals, St. Louis, MO 63178; and 4MU-NAG from Nakarai Chemicals, Kyoto, Japan. p-Nitrophenyl glycosides of β-D-glucose, β-D-galactose, and β-D-glucuronic acid were purchased from Koch-Light Laboratories, Colnbrook, U.K. (All reagents for the colorimetry of urinary NAGase by the MCP-NAG method are commercially available as a kit from Shionogi, Osaka, Japan.

**Urine samples.** We used untimed urine specimens, from among the routine clinical specimens submitted to Shionogi Clinical Laboratories. Urine samples from normal healthy subjects were used to establish the assay conditions.

**Enzyme assay.** In the MCP-NAG method, the substrate solution (1.0 mL, pH 4.90), which contained 2.75 mmol of MCP-NAG and 3.07 mmol of borax per liter of citrate buffer (50 mmol/L, pH 4.75), was preincubated for 5 min at 37°C. A 50-µL urine sample was added and the mixture was incubated for 15 min at 37°C. The reaction was terminated by adding 2.0 mL of 0.3 mol/L sodium carbonate and the absorbance at 580 nm was measured. As the substrate blank, distilled water was substituted for the urine sample.

In the fluorimetric procedure, 0.2 mL of an aqueous 5.27 mmol/L solution of 4MU-NAG was mixed with 0.75 mL of citrate buffer (0.15 mol/L, pH 4.50) and a 50-µL sample of urine was then added. The mixture was incubated for 30 min at 37°C and 2.0 mL of 0.2 mol/L glycine/NaOH buffer, pH 10.5, was then added. The fluorescence intensity was measured at excitation and emission wavelengths of 370 and 445 nm, respectively. As substrate and urine blanks, distilled water was used instead.

Activities of β-glucosidase, β-galactosidase, and β-gluc-
uronidase were determined by using 5 mmol/L solutions of the $p$-nitrophenyl glycosides of each sugar as substrates, according to the MCP-NAG method.

One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 $\mu$mol of the aglycone per minute under the conditions described above.

**Synthesis of MCP-NAG.** Figure 1 outlines the synthesis of MCP-NAG. Acetochloroglucosamine (I), prepared by the method of Leaback (19), was treated with disodium salt of $m$-cresolsulfonphthalein (III) in dimethylformamide to give the tetraacetate (IV), which was acetylated to the pentaacetate (V) for purification. Treatment of V with sodium methylate in methanol yielded MCP-NAG (VI) as a hygroscopic, reddish-orange powder.

**Analysis:** Calcd. for C$_{25}$H$_{18}$NO$_{10}$SNa · 3½H$_2$O: C, 51.93; H, 5.56; N, 2.09; S, 4.78; Na, 3.43; H$_2$O, 9.40. Found: C, 51.71; H, 5.56; N, 2.20; S, 4.93; Na, 3.60; H$_2$O, 9.72.

**Results**

NAGase hydrolyzes MCP-NAG to MCP and N-acetylglucosamine (Figure 2). MCP and MCP-NAG show absorption maxima at 580 and 414 nm, respectively. The released MCP can be measured in alkaline solution at 580 nm by subtracting the absorbance of MCP-NAG substrate blank.

Properties of human urinary NAGase were investigated. The pH is optimum between 4.75 and 5.25 (Figure 3). The reaction proceeds linearly for 30 min at 37 °C and for 20 min at 45 °C (Figure 4). NAGase is inactivated gradually at 50 °C, rapidly at 60 °C. We calculated the $K_m$ value for MCP-NAG to be 0.41 mmol/L from the plot of $S/V_0$ vs $S$ (Figure 5).

Blank values for the MCP-NAG and PNP-NAG methods were compared by using urine samples of light to deep colors (Table 1). The PNP-NAG method gave relatively high urine blanks, which were roughly parallel to color density and in the range of 1.6 to 5.2 U/L equivalent NAGase activity. In contrast, urine blanks in the MCP-NAG method were as low as 0.2 U/L, even for deeply colored samples.

The developed color of the MCP was very stable during the 6-h test period. A typical standard curve (Figure 6) was
Table 1. Urine Blanks Compared between the MCP-NAG and PNP-NAG Methods for Four Urine Samples

<table>
<thead>
<tr>
<th></th>
<th>PNP-NAG method</th>
<th>MCP-NAG method</th>
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<tbody>
<tr>
<td>$A_{405}$ nm</td>
<td>U/L*</td>
<td>$A_{405}$ nm</td>
</tr>
<tr>
<td>0.016</td>
<td>1.6</td>
<td>0.001</td>
</tr>
<tr>
<td>0.026</td>
<td>2.6</td>
<td>0.001</td>
</tr>
<tr>
<td>0.032</td>
<td>3.2</td>
<td>0.002</td>
</tr>
<tr>
<td>0.052</td>
<td>5.2</td>
<td>0.002</td>
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* Equivalent to NAGase activity.

Discussion

A substrate for NAGase assay should have the following
Table 2. Within- and Between-Assay Variations (Three Urine Samples)

<table>
<thead>
<tr>
<th>Within-assay</th>
<th>Between-assay</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
<td>df</td>
</tr>
<tr>
<td>6.25</td>
<td>17</td>
</tr>
<tr>
<td>28.74</td>
<td>17</td>
</tr>
<tr>
<td>33.67</td>
<td>17</td>
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Properties: (a) The aglycone should have an absorption maximum >550 nm, to obviate the effects of colored substances such as bilirubin and hemoglobin. (b) The molar absorptivity of the aglycone should exceed 20 000, to allow a sensitive assay. (c) The substrate should be highly soluble in the reaction buffer, so as to saturate NAGase in the assay mixture.

MCP-NAG is a substrate that satisfies all these criteria. The λ_max and ε for the aglycone MCP are 580 nm and 40 670, respectively. In fact, spectral overlap of interfering substances at 580 nm is almost negligible, so that a urine blank can be omitted in the assay procedure. The high molar absorptivity of MCP gives sufficient analytical sensitivity at a reaction time of 15 min. MCP-NAG is freely soluble in the reaction buffer and shows good reactivity with NAGase. We used a substrate solution of 2.75 mmol/L, which is about seven times the K_m. The initial velocity was estimated to be 87% of V_max. The within-assay and between-assay variations were acceptable, and results correlated well with those by the 4MU-NAG method.

Clearly, our MCP-NAG method has several advantages over conventional methods for determining NAGase activity in the urine, and it may be useful for clinical studies or for other uses in which many samples are to be tested.

References