Rapid Colorimetric Assay of Urinary β -Galactosidase and *N*-Acetyl- β -D-Glucosaminidase With Cobas Mire Auto-Analyzer

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A rapid and simple colorimetric method for the assay of urinary β -galactosidase (GAL) and *N*-acetyl- β -D-glucosaminidase (NAG) using 4-nitrophenyl-glycosides as the substrates and a Cobas Mire Auto-analyzer is described. Optimal conditions for this method including substrate concentration, ionic strength, pH and incubation time were characterized. Endogenous substances in urine

did not interfere with the assays. Incubation time could be shortened to 10 minutes. A small volume of an alkaline buffer to terminate the enzyme reaction achieved better sensitivity and accuracy. Buffer-substrate solutions were stable for at least one week at 4°C. Normal values of the two urinary enzymes are also reported. *J. Clin. Lab. Anal.* 13:95–98, 1999. © 1999 Wiley-Liss, Inc.

Key words: *N*-acetyl- β -D-glucosaminidase; β -galactosidase; urine; automatic-assay

INTRODUCTION

The glycosidases of lysosomal origin in urine has been widely used for the diagnosis of kidney diseases in recent years. Fluorometric method for the determination of urinary GAL and NAG requires fluorometer that is not generally available (1). Kinetic assay of the two enzymes in urine with the substrate 2-chloro-4-nitrophenyl-glycosides was reported (2,3). However, low solubility and instability of the substrate in buffer solution limited its use. In addition, extinction coefficient of the enzymatic product 2-chloro-4-nitrophenol was significantly affected at the pH of the citrate buffer. Therefore, results from this method could not be duplicated perfectly. Though colorimetric method has been described (4), the optimal condition for the assay of GAL and NAG in urine has not been evaluated. We first defined the optimal conditions for urinary GAL and NAG assays using 4-nitrophenyl-glycosides as the substrates on a Cobas Mire Auto-analyser, and thus developed a rapid and simple auto-analysis for the two urinary enzymes suitable for routine clinical usage.

MATERIALS AND METHODS

Sample Collection

Samples from first urinate in the morning from healthy subjects and patients with nephrotic syndrome were collected. Three mL of urine was centrifuged at 600g for 5 min and the supernatant was stored at 4°C no longer than one week.

Instruments and Reagents

We used the Cobas Mire Auto-analyzer (Roche Corp, Basel, Switzerland) for the studies. Buffer pH was measured on pH-

Meter-II (Shanghai, China). For the assay of GAL, 4-nitrophenyl-galactopyranoside (Tianjin Medicine Research Institute, China) was dissolved under gently shaking in 50 mM citrate buffer (pH 4.0 ± 0.05 at 37°C) to 2.5 mM. For NAG determination, 4-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Tianjin Medical Research Institute) was dissolved with gentle warming in 50 mM citrate buffer (pH 4.25 ± 0.05 at 37°C) to 10mM. Buffer-substrate solutions were stored at 4°C for less than one week. Solution should be discarded if reagent blank value exceeded absorbance of 0.10 or 0.30, for GAL and NAG, respectively, under the conditions described below.

Procedures

Mixed 20 μ L urine supernatant and 300 μ L buffer-substrate solution in a cuvette with 0.5 cm light path, incubated the mixture at 37°C for 10 min, and read the absorbance A_1 at 405 nm. The reaction was then terminated by adding 80 μ L of 200 mM borate buffer (pH 12.0) to the mixture, and recorded the absorbance A_2 at 405 nm. The amount of liberated 4-nitrophenol was obtained photometrically at 405 nm from the absorbance difference between A_2 and A_1 against the blanks.

Calculation

Enzyme activity was calculated from extinction coefficient of 4-nitrophenol in alkaline solution (pH 10) at 405 nm (18.5

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$\text{mmol}^{-1} \cdot \text{cm}^{-1}$) and expressed as international unit per liter urine (U/L, 37°C):

$$\begin{aligned} \text{U/L} &= (A_2 - KV \times A_1) / T \times TV / SV \times 1/E \times 1/L \\ &= (A_2 - 0.8A_1) \times 180.2 \end{aligned}$$

(KV, volume correction factor; TV, total incubation volume; SV, sample volume; E, extinction coefficient; L, light path; T, incubation time).

Urinary NAG and GAL activities should be further corrected by creatinine excretion, and expressed as unit per millimole of creatinine (U/mmol Cr).

RESULTS

Optimal Reaction Conditions for the Assays

Incubation time on enzyme activity

We followed the procedure described above except adding borate buffer at different time points, and we diluted the sample if its activity exceeded 200 U/L. Reaction rate was constant with time for GAL for at least 20 min and for NAG for at least 15 min. At the incubation time of 10 min, formation of 4-nitrophenol was proportional to the concentration of GAL and NAG in the mixture.

pH of citrate buffer

All experiment conditions as described in Procedures were fixed except the pH of the buffer-substrate. Optimal pH was in the range of 3.9–4.1 and 4.15–4.35 for GAL and NAG, respectively (Figs. 1 and 2).

Buffer concentration

Varying the concentration of the citrate buffer in the buffer-substrate had influences on GAL and NAG activities. Concentration of citrate buffer higher than 50 mM inhibited GAL and NAG activities (Fig. 3).

Fig. 1. pH of citrate buffer on GAL activity from two urine samples.

Fig. 2. pH of citrate buffer on NAG activity from two urine samples.

Substrate concentration

By using the test condition shown in Procedures, the K_m of GAL for 4-nitrophenyl-galactopyranoside and the K_m of NAG for 4-nitrophenyl-*N*-acetyl- β -D-glucosaminide were defined (Figs. 4 and 5). We therefore used a substrate concentration approximately 10 times higher than the K_m for the assay reactions.

Endogenous Urinary Compounds

Urinary NAG and GAL activities were measured before and after dialysis and showed negligible differences (Table 1). Dilution of the sample with distilled water to 1:4 before test also had no significant changes on GAL and NAG activities.

Accuracy

With-run and day-to-day variation of the same sample were estimated from 5 samples containing 4–150 U/L of GAL and NAG. With-run variation and day-to-day varia-

Fig. 3. Concentration of citrate buffer on activities of GAL and NAG (—●—, GAL; —○—, NAG).

Fig. 4. K_m of GAL for 4-nitrophenyl-galactopyranoside.

tion were $< 3.5\%$ and $< 5\%$, respectively, for both of the enzymes.

Normal Values

Traditionally, a 24-hour urine sample should be collected for the assays to eliminate the fluctuation of urine volume on enzyme concentration in urine. However, this is inconvenient for clinical usage. Here we used the sample from first urinate in the morning to measure GAL, NAG and creatinine, and expressed the activities as U/mmol Cr. Table 2 shows the urinary enzyme activities in samples of first urinate in the morning from healthy individuals of different age groups. Young children appeared to excrete more GAL and NAG in urine. Neonates were found to have the highest activities of both enzymes as compared to other age groups ($P < 0.01$). The group of 1 month to 3 years also showed higher levels than those of the adult group ($P < 0.05$).

Fig. 5. K_m of NAG for 4-nitrophenyl-*N*-acetyl- β -D-glucosaminide.

TABLE 1. Changes of Urinary GAL and NAG Activities After Dialysis

Sample	GAL (U/L)		NAG (U/L)	
	Before dialysis	After dialysis	Before dialysis	After dialysis
1	6.3	6.2	11	13
2	5	4.8	6.8	8
3	3	3.2	7.4	7
4	20	22	100	109
5	18	19	50	17
6	11	10.5	20	22
7	18	19	15	17
8	10	10.5	18.4	18
9	9	9.5	40	39
10	50	49	150	152

DISCUSSION

Manual colorimetric assay for GAL and NAG, which needs to set a sample blank to diminish the interference from urinary color and incubate the mixture for 30 min, is inconvenient and time-consuming. The present auto-colorimetric assay has shown itself to be rapid and simple as compared to the manual method and to be sensitive and accurate as compared to the kinetic method using 2-chloro-4-nitrophenyl-glycosides as the substrate. Due to a greater dilution of the sample in buffer-substrate solution, this method was not affected by endogenous compounds in urine, such as enzyme inhibitors of low molecular weight, and nonspecific catalysts to 4-nitrophenyl-glycosides, such as ascorbic acid. We used 80 μ L of borate buffer to terminate the enzyme reaction, allowing for a pH of 10.5 in the mixture after incubation. Thus, absorbance of 4-nitrophenol in the mixture remained constant for at least 20 min and nonspecific hydrolysis of the substrates was not observed.

The kidney plays an essential role in metabolism. The epithelial cells of renal tubules, especially the proximal convoluted tubules, are rich in enzymes. These enzymes are mainly located in lysosomes and brush borders. There are many kinds of acid hydrolases in lysosomes, including glycosidases, peptidases, and esterases. The brush borders have high contents of alanine aminopeptidase, γ -glutamyltransferase, and alkaline phosphatase. Elevation of lysosomal enzymes in urine suggests renal tubular damage. More than 50 lysosomal enzymes have been identified; however, only those having larger molecular weight (> 80 KD), higher tissue specificity, and being stable in urine and easily measured can be considered for clinical purposes. Urinary GAL and NAG of lysosomal origin have been widely used in the diagnosis and monitoring of renal injuries. Using the present method, we further evaluated the significance of urinary GAL and NAG activities in patients with nephrotic syndrome. Patients with nephrotic syndrome sensitive to Prednisone (4–14 years of age, 4 females and 6 males) showed high levels of NAG and moderate elevations of GAL in urine before treatment in most cases. With the course of remission and the reduction of urinary protein excretion after treatment, NAG in urine

TABLE 2. Urinary GAL and NAG in Samples of First Morning Urinate From Normal Person

Age	Number of persons tested	GAL (U/mmol Cr)		NAG (U/mmol Cr)	
		Range	Median	Range	Median
Neonate	25	1.7–3.2	1.4	0.77–10	4.3
1 month–3 years	80	0.04–2.1	1.19	0.56–4.5	1.93
4–7 years	120	0.05–1.41	0.6	0.57–3.12	1.25
8–14 years	150	0.05–1.19	0.6	0.21–2.47	0.92
15–60 years	100	0.01–1.13	0.45	0.22–2.48	0.91

decreased gradually while GAL in urine first increased then decreased. Both urinary GAL and NAG returned to normal whenever urinary protein became negative and the symptoms remitted completely. Patients with nephrotic syndrome insensitive to Prednisone (4–14 years of age, 5 females and 5 males) showed high levels of NAG in urine with little day-to-day fluctuation and moderate increases of GAL in urine. The explanation for those changes of urinary GAL and NAG may be that NAG is presented in significant quantities in proximal convoluted tubules, while GAL is related to the repair processes of the renal tubules. Damage of proximal convoluted tubules leads to the leakage of lysosomal NAG from the tubular cells into urine. With the recovery of renal damage, GAL presents in nascent proximal convoluted tubules and may leak into urine. Consequently, NAG is probably a sensitive index of tubular damage (5), and GAL may provide information about the repair processes of renal tubules. Measurement of urinary NAG and GAL output may therefore be helpful for the evaluation of renal tissue damages (6).

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