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Sensitive method for the quantification of β-glucuronidase activity in human urine using capillary electrophoresis with fluorescence detection

Xiaojun Wu[a], D. Loganathan[a,c,*], Robert J. Linhardt[a,b,*]

[a]*Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242, USA*
[b]*Department of Chemical and Biochemical Engineering, College of Engineering, University of Iowa, Iowa City, IA 52242, USA*
[c]*Department of Chemistry, Indian Institute of Technology, Madras, India*

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Abstract

Capillary electrophoresis (CE) with fluorescence detection was used to determine the concentration of 4-methylumbelliferone liberated from 4-methylumbelliferyl–β-D-glucuronide by β-glucuronidase. Enzyme substrate saturation kinetics were studied in buffer and the pH range for the enzyme reaction was optimized. A linear relationship of initial enzyme reaction velocity as a function of peak area of enzyme product was obtained for enzyme activity ranging from 1 to 100 units. The β-glucuronidase activity in urine was next determined. Freshly collected urine samples were dialyzed, the retentate was incubated with 4-methylumbelliferyl–β-D-glucuronide, boiled and centrifuged. The supernatant was separated by CE in an uncoated capillary with 0.1 *M* sodium acetate buffer by applying a voltage of 12 kV. The product of the enzymatic reaction, 4-methylumbelliferone, was detected by fluorescence, facilitating the determination of as little as one unit of β-glucuronidase activity in a 0.5-h incubation time, with an error of less than ±5%. © 1998 Elsevier Science B.V.

Keywords: β-Glucuronidase; Enzymes; 4-Methylumbelliferone

1. Introduction

Capillary electrophoresis (CE) is becoming popular in analytical applications due to its advantages over other traditional assays. However, it is still a challenge to overcome many of the problems associated with its use in clinic diagnosis. Human β-glucuronidase (EC 3.2.1.31) is an acid hydrolase that is responsible for lysosomal degradation of conjugated glucuronides. It has recently found important diagnostic uses, such as in lysosomal storage disease [1,2], cancer [3,4] and in human immunodeficiency viral infections [5]. An inherited deficiency of β-glucuronidase activity causes the lysosomal storage disorder mucopolysaccharidosis type VII (Sly disease), where the sequential catabolism of glycosaminoglycans in lysosomes is blocked and undegraded substrates accumulate in the cells of many tissues, including the neurons and glia of brain [2]. A significant increase in the activity of β-glucuronidase has been observed in postmortem gray matter of late infantile and juvenile brains when compared to

*Corresponding authors.

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controls [6]. In the development of sizable tumors, β-glucuronidase activity can be detected histochemically [3]. At the same time, urinary β-glucuronidase activity represents a marker for cancer of the urinary tract and other organs [4]. Enzyme activity is generally higher in the urine of cancer patients than in that of control subjects.

In all of these examples, sensitive and accurate determination of the enzyme activity is pivotal for the development of diagnostics and to assess the effects of this enzyme. Commonly used assays for β-glucuronidase activity include spectrophotometric assays that rely on *p*-nitrophenyl–β-glucuronide and phenolphthalein–β-glucuronide [7,8] as substrates. 4-Methylumbelliferyl–β-D-glucuronide (MUG) has been used in fluorescence-based β-glucuronidase assays [9]. These assays all require a separation process prior to the determination of glucuronidase activity in biological samples. Recently, a high-performance liquid chromatography (HPLC) method was introduced that relied on the liberation of 4-methylumbelliferone (MU) from MUG substrate as an assay for β-glucuronidase activity in biological samples [10]. Here, we report a sensitive CE-based assay that relies on the same substrate to determine β-glucuronidase activity in urine samples.

2. Experimental

2.1. Materials

Bovine liver β-glucuronidase (3.2.1.31) (type-1, 666 400 U/g, modified Fishman units, where 1 U liberates 1 μg of phenolphthalein from phenolphthalein glucuronide in 1 h at 37°C), 4-methylumbelliferyl–β-D-glucuronide (MUG) and 4-methylumbelliferone (MU) were purchased from Sigma (St. Louis, MO, USA). A Microcon-10 microconcentrator with a 10 kDa molecular weight cut-off (MWCO) was from Amicon (Beverly, MA, USA). Spectra/POR molecular porous membranes with a MWCO of 6–8 kDa were from Spectrum Medical Industries (Los Angeles, CA, USA). All chemicals were of analytical grade. Glass distilled (3×) water was used in all experiments.

2.2. Urine sample pretreatment

Freshly voided urine samples from healthy male adults were collected. Samples were treated using a 6–8 kDa MWCO dialysis membrane against 1 m*M* sodium phosphate buffer (pH 6.5) at 4°C for 24 h. Alternatively, a 0.5-ml urine sample was buffer-exchanged and concentrated to 20 μl by centrifuging (at 15 000 *g*) it with Microcon-10 in 10 kDa MWCO microconcentrators in a minicentrifuge (National Labnet, Woodbridge, NJ, USA) at 4°C for 2 h. To the concentrated samples, 200 μl of 100 m*M* sodium phosphate buffer (pH 6.5) was added and the samples were again centrifuged in the Microcon-10 for another 2 h under the same conditions. The concentrated urine sample was finally adjusted to a volume of 200 μl by adding the sodium phosphate buffer.

2.3. Enzymatic reactions and standard solutions

Stock standard solutions of MU and MUG were prepared in 0.1 *M* sodium phosphate buffer (pH 6.5) at concentrations of 0.1 and 10 mg/ml, respectively, and stored at −20°C. Bovine liver β-glucuronidase was freshly prepared before use in the same buffer at 1000 U/ml.

A calibration curve was constructed by adding increasing amounts of β-glucuronidase solution (1 to 100 U) to 50 μl of MUG and making the final volume up to 200 μl with 0.1 *M* sodium phosphate buffer (pH 6.5). The reaction mixture was incubated for 1 h at 37°C in a water bath, after which, the enzyme was denatured by boiling for 5 min. A calibration curve was obtained following CE analysis by plotting the area of the peak corresponding to MU as a function of enzyme units.

2.4. CE conditions

The Dionex CE system (Dionex, Sunnyvale, CA, USA) was linked to an IBM PC computer interface. Gravity sample injection mode relied on a 55-mm height for 30 s. The capillary, 52 cm×50 μm I.D. (injection to detector window), was from Polymicro Technologies (Phoenix, AZ, USA) or Yongnian Capillary Factory (Yongnian, Hebei, China). CE was performed under normal polarity using 100 m*M*

sodium acetate buffer (pH 5.0) and a voltage of 12 kV. Fluorescence detection used an excitation wavelength of 320 nm, with a 375-nm cut-off emission filter.

3. Results and discussion

3.1. Separation and detection by CE

The structure of the MUG substrate and the MU product is shown in Fig. 1. The absorbance maximum for MU and MUG is at 320 nm and this wavelength was used for fluorescence excitation. A 375-nm cut-off emission filter was used for the determination of the fluorescence intensity for MU and MUG.

A representative electropherogram of MUG and MU peaks obtained after a 1-h incubation of a urine sample in which MUG was used as a β-glucuronidase substrate is shown in Fig. 2. Only two well-resolved peaks are observed in this electropherogram, making it easy to distinguish the product peak from the substrate peak. The fluorescence intensity of MU is about 30-times stronger than that of the MUG.

Because of the higher fluorescence sensitivity for detection of the product, MU, the MU peak was chosen to assess enzyme activity. A linear relationship of peak area integration as a function of the concentration of MU (1 to 100 μg) was obtained. As little as 5 nl of a 6-n*M* solution of MU, corresponding to 0.03 fmol, could be detected by this method. This result compared favorably to a reported HPLC detection limit of 20 n*M*, which was obtained using a 100-μl injection volume (corresponding to 2 pmol) [10].

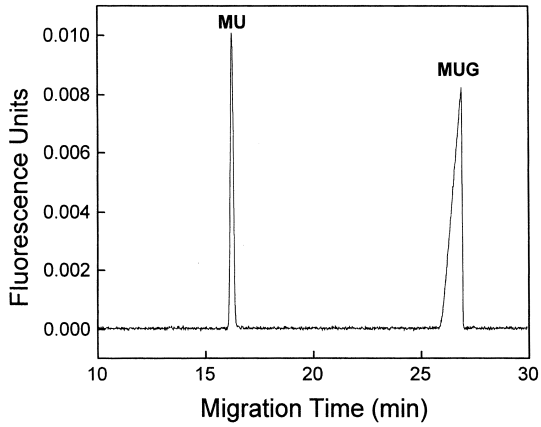

Fig. 2. Electropherogram of MU and MUG peaks during the enzymatic reaction. Enzyme reaction conditions: 200 μg (0.57 μ*M*) of MUG and 20 U of β-glucuronidase in 0.1 *M* sodium phosphate buffer (pH 6.5); total volume, 200 μl; enzyme reaction time, 1 h at 37°C. CE conditions: 52 cm×50 μm I.D. (injection to detector window) capillary; gravity injection, 55 mm height, 30 s; buffer, 0.1 *M* acetic acid–sodium acetate, pH 5.0; running voltage, 12 kV; fluorescence detection with an excitation wavelength of 320 nm and a 375-nm cut-off filter.

3.2. Enzyme kinetics

Enzyme kinetics were studied by changing the amount of MUG substrate in the presence of a fixed amount (100 U) of enzyme to obtain the optimum reaction conditions. The initial velocities for different amounts of MUG were determined by plotting the enzyme reaction time as a function of MU peak area (not shown). From five such curves, the initial velocity of each was obtained. The initial velocity was then plotted as a function of the MUG concentration to obtain information on the substrate saturation of enzyme (Fig. 3). The addition of 500 μg of MUG (1.42 μ*M*) saturates 100 U of β-

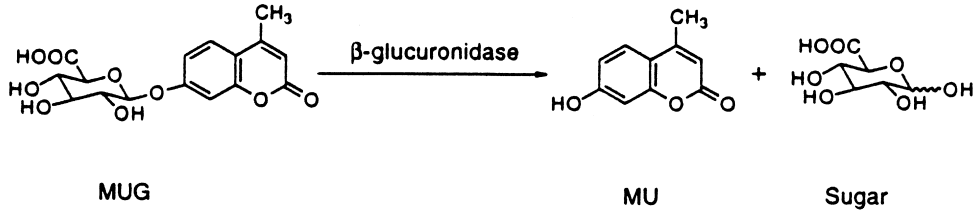

Fig. 1. Conversion of substrate MUG to the products MU and D-glucuronic acid through the action of β-glucuronidase.

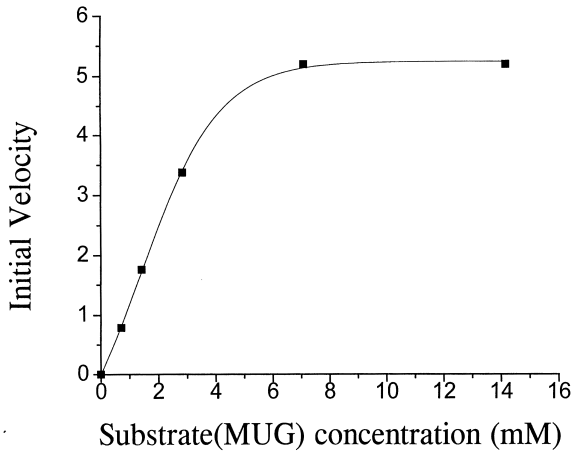

Fig. 3. Enzyme saturation curve. The β-glucuronidase activity was 100 U and the MUG concentration was varied from 0 to 15 m*M*. Other conditions were the same as given in Fig. 2.

glucuronidase. Kinetic analysis shows Michaelis–Menten-type kinetics, with a K_m value of 2.1 m*M*, which is comparable to the value of 1.77 m*M* that was reported for the same enzyme–substrate pair using an HPLC-based assay [10].

3.3. Optimization of assay pH and buffer

The optimum pH for the enzyme reaction had been reported as ranging from pH 5.0 to 6.5 [4]. Freshly voided urine from individuals on normal diets averages about pH 6.0, with a range of pH values from pH 4.5 to slightly higher than 8.0 [11]. Thus, the use of slightly acidic conditions are favorable in developing an assay for β-glucuronidase in urine. It was observed, however, that the optimum pH for the enzymatic reaction was different in different buffer systems. In 0.1 *M* sodium phosphate buffer from pH 5.5 to 7.5, a broad optimum was observed at pH 6.5. In 0.1 *M* sodium acetate buffer, the concentration of MU product increased from pH 4.0 to 5.5.

The pH and buffer systems were also examined under the actual assay conditions. While using CE to separate and analyze MU and MUG, it was found that the intensity of the MU peak measured by fluorescence detection was 50% higher in 0.1 *M* sodium acetate buffer (pH 5.0) than in 0.1 *M* sodium phosphate buffer (not shown). Since the resolution of product and substrate peaks was also excellent under these conditions, 0.1 *M* sodium acetate (pH 5.0) was used in subsequent studies.

3.4. Kinetic determination of enzyme activity in buffer

Kinetic assays using CE were next applied to determine the enzyme activity. The initial velocity of the enzyme reaction was determined in the presence of varying amounts of enzyme (1 to 100 U) by measuring the change in the peak area of MU during the first 0.5 h of the reaction (Fig. 4). A plot of the initial velocity as a function of added enzyme (1 to 100 U) gave a linear curve. A linear relationship was also found between the MU peak area and the enzyme reaction time (from 1 to 24 h) when only 1 U of enzyme was used. While a minimum of 1 U of enzyme could be determined using a 0.5-h incubation time, a prolonged incubation time for the enzyme reaction should be favorable for the highly sensitive determination of very small amounts of the enzyme.

3.5. Kinetic determination of enzyme activity in urine

A linear standard curve was obtained on plotting MU peak area as a function of the amount of liver β-glucuronidase added in the presence of 100 μl of urine that had previously been dialyzed overnight and denatured by heating for 5 min at 100°C (Eq. (1)). The activity (expressed in units for a 1-h assay) of liver β-glucuronidase added to the urine sample was obtained using Eq. (1) by measuring the MU peak area.

$$y = 3004x - 2676 \ (r = 0.992) \quad (1)$$

Here, y is the MU peak area after incubation for 1 h at 37°C and x is the amount of β-glucuronidase added (from 1 to 100 U). These results show that, following dialysis and thermal denaturation, urine components did not interfere with the CE-based assay.

A precision test was done for the CE assay by repeating the determination of the MU peak area after incubation for 24 h at 37°C in the presence of 1 U of added β-glucuronidase and 500 μg of MUG

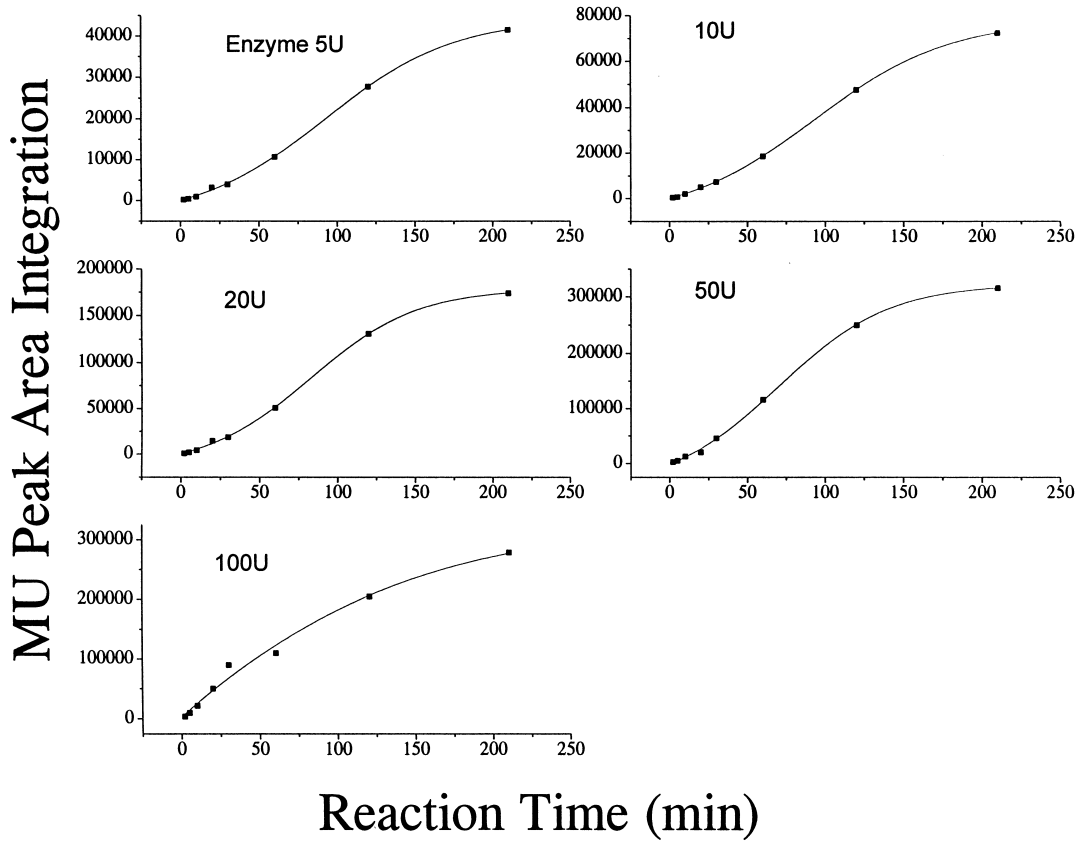

Fig. 4. Relationship between enzyme reaction time and MU peak area. The amount of MUG was 500 μg (1.42 μ*M*) and the β-glucuronidase activity was varied from 5 to 100 U through the addition of bovine liver enzyme; total volume, 200 μl. For reaction and CE conditions, see Fig. 2.

substrate. Statistical treatment [12] of the data resulted in a mean (μ) of 13.25±0.45 μg/ml and a standard deviation of 0.78. Student's *t*-test gave a $t_{0.95} = 1.812$, when the number of degrees of freedom (df) was ten. The accuracy for the measurement of enzyme unitage in urine was also performed using nine independent determinations of endogenous β-glucuronidase contained in a single urine sample. A mean (μ) of 10.3±0.48 μg/ml and a standard deviation of 0.182 was obtained. Thus, the error involved in the CE assay is less than ±5%, meeting the requirements of most analytical applications for measuring β-glucuronidase activity.

Freshly collected urine samples from various healthy male subjects were analyzed in triplicate for β-glucuronidase activity following dialysis for 24 dialysis at 4°C using a 6–8 kDa MWCO membrane (Table 1). Control experiments were performed by thermally denaturing the urine samples (5 min at 100°C) after overnight dialysis. No enzyme activity was detected in the urine samples in the control

Table 1
Measured enzyme activities for different urine samples

Sample[a]	β-Glucuronidase activity (U/ml)
KB	8.8
IC	14.7
MK	28.4
RN	14.4
XJ	17.3, 6.0[b]

[a]An average of four determinations was used for the XJ sample; all other samples are the average values of three determinations.
[b]This urine sample was washed using Amicon-10 followed by centrifugation (twice) with 0.1 *M* sodium phosphate buffer, pH 6.5, and then was diluted to 200 μl for a duplicate determination.

experiments. It was also observed that no enzyme activity could be detected in urine samples that had not be pretreated using dialysis or a microconcentrator. One reason is the possible presence of a specific, dialyzable, β-glucuronidase inhibitor, glucaro-1,4-lactone, in these urine samples [4].

The β-glucuronidase activity of a single urine sample differed depending on whether dialysis or centrifugation was used for sample pretreatment (Table 1). The enzyme activity of urine following sample pretreatment by dialysis was three times greater than that of the sample that had been buffer-exchanged and concentrated by centrifugation. This result suggests that the enzyme may be adsorbed and lost on the membrane or the inner surface of the microconcentrator during high speed centrifugation. Dialysis, therefore, was the method of choice for the pretreatment of urine samples.

3.6. Conclusions

In summary, a convenient, sensitive, precise and accurate CE-based assay has been developed for β-glucuronidase in urine. This assay might play a role in developing new diagnostic methods and for understanding the biological role of β-glucuronidase activity in various disease processes.

Acknowledgements

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