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Short communication

Analysis of urinary *N*-acetyl-β-glucosaminidase by capillary zone electrophoresis

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Abstract

N-Acetyl-β-glucosaminidase (NAG), a glycosidase enzyme, present in serum, urine and the renal lysosomes is utilized clinically as an early marker for renal damage preceding the elevation of both blood urea nitrogen and creatinine. NAG is analyzed by CE after incubation of urine samples with the synthetic substrate methylumbelliferyl-β-D-glucosaminide. The reaction mixture is introduced directly into the instrument without further treatment. The released reaction product, 4-methyl-umbelliferone, is separated at 13.2 kV in a 400 m*M* borate buffer, pH 8.1. Detection was achieved with either ultraviolet absorption or with fluorescence. The fluorescence detection was more sensitive and gave cleaner electropherograms. The CZE method correlated well with an automated kinetic fluorescent assay. 4-Methyl-umbelliferone conjugated to different substrates is used in the analysis of many enzymes involved in the inborn errors of metabolism.

Keywords: Enzymes; *N*-Acetyl-β-glucosaminidase

1. Introduction

Enzymes which hydrolyze polysaccharides are quite common in animal tissues, especially in the lysosomes [1,2]. Several of those enzymes are involved in many of the lysosomal storage disorders such as Tay-Sachs, Sandhoff disease, and Sanfilippo syndrome [1,2]. They are also used clinically as markers in the study of diabetes [3]. *N*-Acetyl-β-glucosaminidase (EC 3.2.1.30) (NAG) is an example of such enzymes. It is a glycosidase found in many mammalian tissues, including solid organs, hematopoietic elements and several body fluids such as serum and urine [4]. The renal tubules are especially rich in this enzyme.

Normally, very small amounts of NAG are present in the urine, rising markedly even after mild renal damage or malfunction, such as that seen after drug intake. Due to the normal kidneys' excess capacity to maintain homeostasis, urinary NAG elevation precedes changes in blood urea nitrogen or creatinine. Elevations of this enzyme are detected in early transplant rejection, diabetes, hypertension, nephrotoxicity due to drugs and other agents, and autoimmune diseases such as Goodpasture's syndrome [5,6].

The analysis of the lysosomal enzymes is difficult. Usually, it depends on utilizing synthetic substrates conjugated to 4-methylumbelliferone or p-nitrophenol derivatives which fluoresce or produce a color upon release from the substrate [1]. Here, we describe a simple method for analysis of such enzymes, using NAG as an example, based on capillary zone electrophoresis (CZE). The assay

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measures NAG activity using either the absorbance at 214 nm or the fluorescence of 4-methylumbelliferone (4-MU) released from the substrate methylumbelliferyl-β-D-glucosaminide (MUG) after separation by CZE.

2. Experimental

2.1. Chemicals

All chemicals were obtained from Sigma Chemicals, (St Louis, MO, USA)

2.2. Enzyme assay

Substrate: 4-methylumbelliferyl-β-D-glucosaminide (300 mg/l) dissolved in a citrate–phosphate buffer (stable for 24 h refrigerated).

Citrate–phosphate buffer: this buffer contained, as final concentrations, both 25 mmol/l citric acid and 75 mmol/l NaH_2PO_4 and adjusted, if necessary to pH 4.5 (stable for 3 months refrigerated).

2.3. Procedure

Urine (5 μl) was incubated with 200 μl of substrate buffer for 1 h at 37°C. The reaction was stopped by placing the samples on ice.

2.4. CZE

(A) Absorbance: a Model 2000 (Beckman Instruments, Fullerton, CA, USA) equipped with a capillary 42 cm×50 μm was set at 214 nm and 13.2 kV. The running buffer was boric acid, 400 m*M* adjusted to pH 8.1 with NH_4OH. The capillary was rinsed for 2 min with NaOH 0.2 *M*, followed by 2 min with the electrophoresis buffer. Samples were injected for 7 s and electrophoresed for 5 min.

(B) Fluorescence: a home-made CE instrument was used mainly for demonstration purposes of the sensitivity and the lack of interference. A Model EL power supply (Glassman, Whitehouse Station, NJ, USA) and a model Spectra/glo (Gilson Medical Instruments, Middleton, WI, USA) with an excitation wavelength at 365 nm and an emission filter at 455 nm. Because this fluorometer is not designed for CZE work and lacks sensitivity, we used a wider capillary, 200 μm×60 cm I.D. (28 cm to the detector) which also facilitates buffer aspiration. The

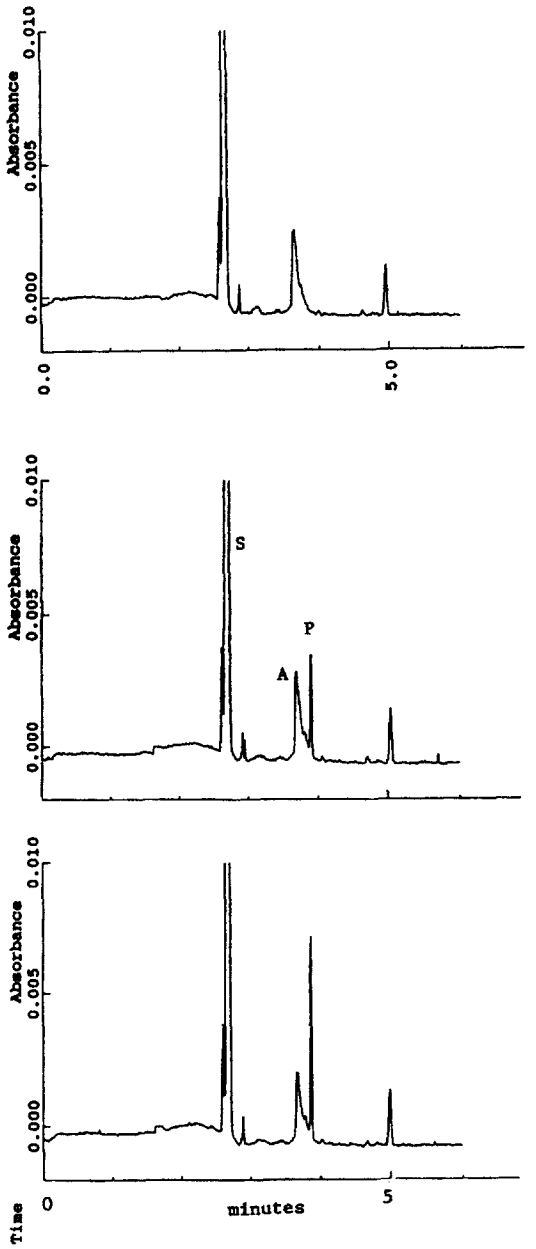

Fig. 1. Urine (enzymatic activity: 36 U/l) after incubation with the substrate (monitored at 214 nm) for: 0 min (top), 30 min (middle); and 60 min (bottom). (S=substrate, P=4-methylumbelliferone, A=albumin).

capillary was filled with buffer (100 mmol/l boric acid adjusted to pH 8.1 with sodium hydroxide). Samples were introduced by gravity (10 cm height) for 5 s and electrophoresed for 6 min at 9 kV.

2.5. Automated kinetic fluorescence

An automated commercial centrifugal analyzer which delivers the substrates in special cuvettes, mixes, incubates, measures the increase in fluorescence and calculates the results, as described earlier, was used for comparison with the CE [6].

2.6. Calculation

Enzyme activity was based on peak height using a secondary calibrator [7].

3. Results and discussion

After incubating the urine with substrate MUG, the enzymatic product 4-MU is released, separated from other compounds in the sample and detected by absorbance at 214 nm, Fig. 1. As the incubation time increases, there is a proportional increase in the 4-MU peak height (Fig. 1 – bottom). By incubation of an elevated sample (34 U/l), for different periods of time, the increase in absorbance (enzymatic activity) was linear as measured by peak height for about 90 min (Fig. 2). We used a long reaction period (60 min) in order to decrease the variability due to the incubation time. The migration time of 4-MU was about 4 min. After spiking the incubated samples with pure 4-MU, the peak height of the

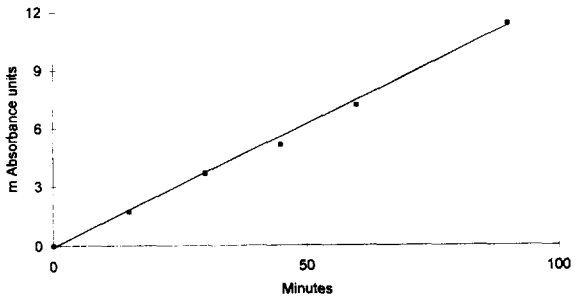

Fig. 2. Linearity of the reaction time for a sample of 34 U/l. The sample was incubated for different periods of time.

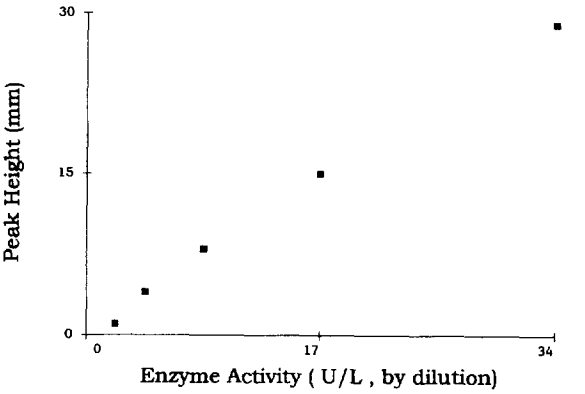

Fig. 3. Linearity of dilution of a sample with elevated activity (36 U/l).

released product increased further indicating that the released product is 4-MU. The substrate itself (MUG) migrated with the neutral compounds. A decrease in the peak height of MUG can be noted after the reaction, especially when kidney tissues are assayed. Thus, not only urine but tissues can be analyzed by the same method after homogenization in the citrate buffer. However, in the case of the urine samples, many other molecules co-migrate with the substrate.

It is known that urine contains many compounds which can interfere in the manual fluorimetric assay in general [6]. Some of these compounds are direct inhibitors of NAG while other compounds quench the fluorescence [8]. Using a high ratio of reagent to

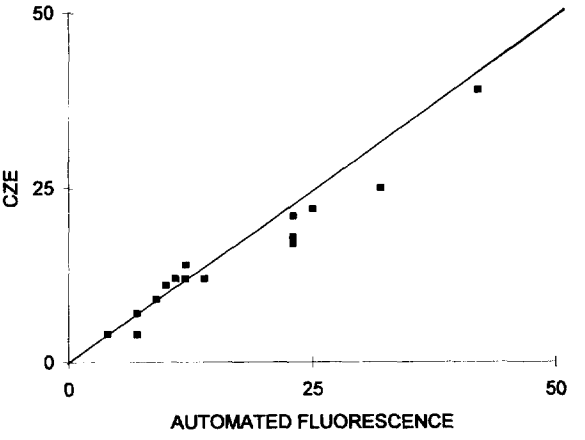

Fig. 4. Comparison of the CZE method with kinetic automated fluorimetric assay (6), (r=0.97, n=15).

urine (40:1), as in this procedure, decreases the effect of the inhibitors [8]. To check for the reaction linearity, a high sample (36 U/l) was diluted in water, (Fig. 3). It showed a good linearity from 1.4–23 U/l (Fig. 3) indicating that the peak height (absorbance) is linearly related to enzymatic activity (concentration) and also indicating the absence of inhibitors. Utilizing absorbance detection in CZE eliminates the quenching effect. The possibility of interference by drug administration and other urinary constituents was evaluated by assaying four urine pools from hospitalized patients. No interfering

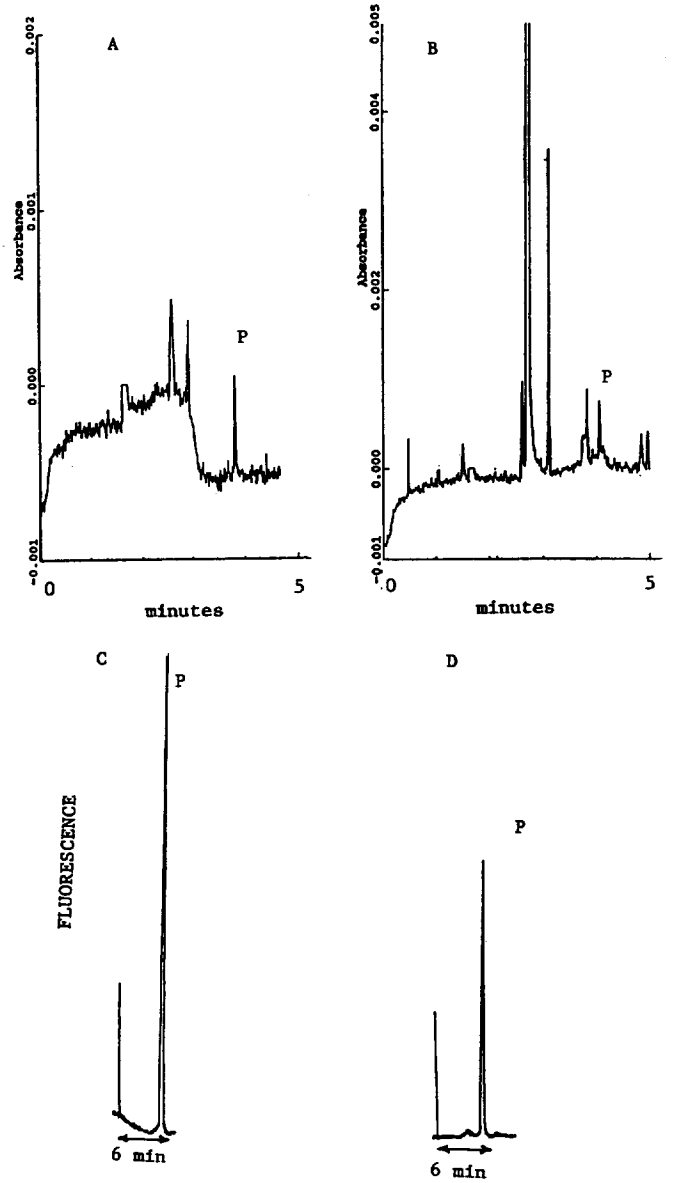

Fig. 5. Comparison of CZE by absorbance at 214 nm (top) and fluorescence (bottom). (A) Aqueous standard 1 mg/l of 4-methylumbelliferone (P) by UV absorbance at 214 nm. (B) Urine low activity (3 U/l) at 60 min by UV absorbance at 214 nm. (C) Aqueous standard 1 mg/l of 4-methylumbelliferone by fluorescence. (D) Same urine as in (B) at 60 min by fluorescence.

peaks were identified. Albumin, which can be detected in some urine, migrates as a wide peak close to that of 4-MU but can be easily recognized, Fig. 1—middle. A high buffer concentration is required to keep the peak of albumin migrating slightly ahead of 4-MU peak.

This CZE method compared well to a kinetic fluorescence assay [6], (r=0.974), Fig. 4. In order to convert peak height (area) to enzymatic activity (U/l) we used a secondary calibrator, i.e., a sample analyzed by the kinetic method as a standard [7].

Since the 4-MU also fluoresces, we evaluated the analysis of NAG activity by a laboratory-made CZE with fluorimetric detection. Because the detector utilized in this work is not designed for CZE or for sensitive analysis, we used a wider capillary, 200 μm (I.D.). Under these conditions, less interference and greater sensitivity (better signal-to-noise ratio) is observed by fluorimetric detection leading to very clean electropherograms, Fig. 5D compared to absorbance at 214 nm, Fig. 5B. Proteins and the majority of small molecules are not detected by fluorescence. A small peak is detected by fluorescence in the blank which arises from the substrate itself. This peak tends to increase in height upon storage of the substrate. Because of the high sensitivity of the fluorimetric method it is suited for analysis of enzymes with low activity.

Capillary electrophoresis offers specific advantages in the analysis of enzymes [9]. The incubation step can be accomplished inside or outside of the capillary [9]. An incubation outside the capillary results in peaks which are easy to quantitate. Previously we have shown that CE is well suited for the analysis of proteolytic enzymes [7]. Here, we demonstrate that an important group of the hydrolytic enzymes which are involved in assessing renal damage and many of the inborn errors of metabolism, and which are difficult to analyze, can be measured by CE. These enzymes can be measured by absorbance or by fluorescence detection. The fluorescence analysis is more sensitive and yields cleaner electropherograms.

In summary, the CZE assay for NAG activity is simple, rapid, and inexpensive, and can provide early detection of renal damage before the usually recognized parameters of renal function have changed.

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