Rapid Assay of N-Acetyl-β-D-glucosaminidase Isoenzymes in Urine by Ion-Exchange Chromatography

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We describe a new method for separating and measuring urinary N-acetyl-β-D-glucosaminidase isoenzymes by "high-performance" liquid chromatography. Isoenzymes are eluted from the anion-exchange column with a one-step linear gradient of NaCl solution. For continuous post-column quantification of the activities of these isoenzymes, we use an online post-column reactor and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as substrate; the methylumbelliferone formed in this reaction is quantified fluorimetrically. We discuss the effects of varying different components of the assay: NaCl concentration, the pH of the mobile phase and of the reaction reagent, substrate concentration, incubation temperature, and the geometry of the post-column reactor.

Additional Keyphrases: fluorometry · post-column reactors

The glycohdyrolase N-acetyl-β-D-glucosaminidase (NAG; N-acetyl-β-D-glucosaminide N-acetylglucosaminidase, EC 3.2.1.30) is widespread in the animal kingdom.3 High concentrations are present in kidney and in lysosomal subcellular fractions (1, 2). In urine from healthy humans, this enzyme is composed of two major isoenzymes (3): one with an acidic isoelectric point (A form) and one with a basic isoelectric point (B form). The latter may represent either a metabolic precursor or a possible degradation product of the A form and has a lower content of N-acetylenuraminic acid than the A form does (4). Slight traces of minor components (I1 and I2 forms) are present in urines of patients with renal disease (3, 5) or of patients who have rejected renal transplants (6); an additional isoenzymic A* form has also been described in urine samples containing blood (3) and in urine from patients with predominantly glomerular disorders (7).

In previous reports, activities of NAG isoenzymes were measured after separation of the isoenzymes by ion-exchange chromatography on diethylaminoethyl cellulose; the activities were determined either in eluate fractions (3, 8) or by on-line monitoring of column effluent (7, 9). Because these procedures are relatively slow, we became interested in the possibility of using continuous "high-performance" liquid chromatography (HPLC). Indeed, the HPLC method allows repeated use of one column; better control over chromatographic conditions, because it includes equipment that can generate closely controlled flow rates with mobile-phase gradients; and improvement of analysis time, owing to the use of rigid packings, which permit high flow rates with little loss of chromatographic efficiency.

Here we describe the use of HPLC with an anion-exchange column to separate the urinary NAG isoenzymes and their quantification by an on-line post-column reaction system.

Materials and Methods

Apparatus

The HPLC equipment consisted of two Model 6000 A pumps and an automated gradient controller (all from Waters, Paris, France), a Rhodyne 7125 injection valve (Touzart et Matignon, Vitry-sur-Seine, France) equipped with a 250-μL loop, and a Model F 1000 Hitachi spectrofluorimeter (E. Merck, Darmstadt, F.R.G.). The detector output was connected to a C-RSA Shimadzu integrator (Touzart et Matignon). The 50 × 5 mm (i.d.) column was packed with Mono Q HR 5/5 anion-exchange resin (Pharmacia, Uppsala, Sweden), a hydrophilic resin (average particle diameter 10 μm) on which the charged group is —CH2—N(CH3)3.

We used two Eldex A-30-S 2 pumps (Bioblock, Paris, France) for post-column reagent (substrate and pH 11.0 buffer) pumps. The column effluent was mixed with substrate reagent, and the reaction mixture was mixed with pH 11.0 buffer by passing through T-connectors (Touzart et Matignon). The enzymatic reaction was carried out in a 50 m × 0.50 mm (i.d.) coiled Teflon tubular reactor (CTR) kept at 50 °C in a water-bath; the coil diameter was 4 cm. For comparison we used a packed-bed reactor consisting of a 1 m × 4.6 mm stainless-steel column packed with 5- to 10-μm diameter glass beads. With this latter system it was necessary to saturate the glass beads, whether silanized or not, with albumin by injecting 20 mg of bovine albumin into the reactor before each day’s use. For pretreatment of urines by gel-filtration we used a peristaltic Minipuls II pump (Gilson, Villiers le Bel, France) and monitored results with an ultraviolet spectrophotometer (Uvicord II; LKB, Orsay, France).

Reagents

4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide was purchased from Sigma Chemical Co., St. Louis, MO. Tris, sodium citrate, and sodium glycinate (all "p.a." grade) were from Merck; Sephadex G-50 Superfine was from Pharmacia.

Mobile phases were prepared as follows. Solution A (Tris, 10 mmol/L): dissolve 1.21 g of Tris in about 500 mL of distilled water, adjust to pH 7.0 by adding hydrochloric acid, and dilute to 1 L with distilled water. Solution B (0.5 mol/L NaCl in Tris buffer): dissolve 29.25 g of sodium chloride in 1 L of solution A. Stored at 4 °C, these solutions were stable for a week.

Prepare fresh substrate solution (0.93 mmol/L) daily by diluting 72 mg of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide in 200 mL of sodium citrate buffer (0.2 mol/L, pH 4.8).
Urine Specimens

The 20-mL aliquots of 24-h urine specimens from renal transplant patients were stored without preservatives at −20 °C in plastic flasks and examined within a month of collection. Before assay, we thawed 1 mL of urine, applied it to a 5 × 1.5 cm (i.d.) glass column containing Sephadex G-50 gel, and eluted with solution A. We monitored the absorbance of the column effluent at 280 nm, and collected 4 mL of the eluate corresponding to the first peak, which contained the enzymes. This desalting procedure separates proteins from salts and from compounds of low relative molecular mass (Mr < 1500) that could inhibit enzyme reaction (10).

Chromatography

The conditions for the one-step linear gradient elution (1 mL/min) were as follows: starting eluent, 20% solution B (80% solution A) for 5 min, increasing linearly to 45% solution B in 25 min, then returning to the starting conditions in 5 min and holding there (to equilibrate the anion exchanger) for 5 min before the next sample (150 μL of pretreated urine) was injected.

The column effluent was mixed by means of a T-connector with the substrate solution, which was pumped at a flow rate of 0.5 mL/min and incubated in the CTR at 50 °C. The reaction time was fixed by the flow rate through the CTR and the internal volume of this reactor. We used another T-connector, placed at the end of the CTR, to add sodium glycinate buffer (0.2 mol/L, pH 11.0) to the CTR effluent stream at a flow rate of 0.5 mL/min. This alkaline buffer both stopped the reaction and increased the fluorescence of the 4-methylumbelliferone formed during the incubation period (11). We quantified the released 4-methylumbelliferone fluorimetrically at λex = 365 nm and λem = 445 nm.

Measuring Enzyme Activities

We found that the detector response varied linearly with the amount of 4-methylumbelliferone injected, from 5.68 to 141.88 pmol; so, in this range, we calculated the quantities of 4-methylumbelliferone formed during the isoenzyme reaction by comparing each peak area with the peak area corresponding to the injection of 62.5 pmol of this compound. We then expressed NAG isoenzyme activity as micromoles of 4-methylumbelliferone released from the substrate per hour per litre, and expressed urine isoenzyme activity relative to creatinine concentration as units of NAG isoenzyme activity per micromole of creatinine. We checked the calibration daily, by analyzing an aliquot of a previously assayed urine sample stored at −20 °C.

Results and Discussion

Chromatographic Separation of NAG Isoenzymes

Figure 1 shows three chromatograms from urine samples of renal transplant patients who had no clinical or biological evidence of organ rejection. This elution order of NAG B and NAG A is the result of our use of an anion-exchanger resin with a mobile phase at pH 7.0. Because NAG B has a basic isoelectric point, this isoenzyme is eluted at a lower salt concentration than is NAG A. Also, we were not able to detect with certainty the elution of L1, L2, or A isoforms, but if the N-acetylhexosaminic acid content of these isoenzymes is intermediate between NAG A and NAG B (4), we would expect these minor fractions to be eluted between the well-resolved peaks for NAG A and NAG B. Samples can be injected at 35-min intervals.

Analytical Validation of Optimized Assay Conditions

Sample collection and preparation of urine for assay. Wellwood et al. (10) assumed that a urine sample taken at any time during the day could be used to represent daily NAG excretion, if the effect of different rates of urine flow was eliminated by relating NAG activity to creatinine output. However, because of evidence of a circadian rhythm in urinary N-acetyl-β-D-glucosaminidase excretion, with peak excretion 11 h after the maximum excretion of urinary creatinine (12), we prefer to collect 24-h urines. To avoid changes in NAG activity caused by the presence of bacteria or certain preservatives (10), we collected urines in sterile plastic flasks and stored these at −20 °C for no more than one month. Some investigators noted the highest NAG activities in urines diluted 20- or 40-fold (10, 13) but decreases in activity at greater dilutions (10). Dialysis of urine against distilled water increases NAG activity slightly (13), but we prefer using gel filtration of urine, as described by Maruhn (14), to remove inhibitors of enzyme activity. Maruhn (14) found that NAG activities measured after gel filtration were 19% higher than before; in addition, gel filtration is generally faster than dialysis.

Gradient elution. Because acetate anions are well-known endogenous inhibitors of NAG activity (15), chromatographic separation of NAG isoenzymes was obtained with sodium chloride. The decreases shown in Figure 2 agree with those reported by Chow and Weissmann (16); to minimize this decrease, we used a maximum salt concentration of 225 mmol/L in the mobile phase. Because NaCl corrodes stainless steel tubing, we thoroughly flushed the chromatographic system with 50 mL of distilled water after each day's use.

pH of reaction mixture and of mobile phase. In agreement with Leaback and Walker (17), we noted that the pH of the reaction mixture affected the enzyme activity, with maximum activity at pH 4.8 (Figure 3). Figure 4 illustrates the influence of pH of the mobile phase on the pH of the reaction mixture, on the enzyme activities, and on resolution. There was negligible effect on the pH of the reaction mixture when eluent pH varied from 6.0 to 7.5; isoenzyme activities and separation were greatest at pH 7.0.

Substrate concentration. Because of the high sensitivity of the fluorimetric detection, we could examine the effect of the substrate concentration (17) over a wide range. Given the limited residence time of the reaction mixture in the CTR, and considering that only a small proportion of the substrate should be hydrolyzed (17), we chose as the upper limit of substrate concentration the value previously determined with two urine specimens (Figure 5): 0.93 mmol/L.

Reaction temperature. In our effort to increase detector sensitivity, we examined the effect of temperature on isoenzyme activities. The activity of isoenzyme A reportedly decreased in urine heated at 50 °C for 1 to 4 h (8), and was accompanied by an increase of intermediate (I1 and I2) and B isoenzymes. In our experiment, the activity of isoenzymes A and B was maximum at 50 °C; increasing the reactor temperature from 50 to 70 °C resulted in a rapid, substantial loss of the A and B activities (Figure 6). One reason why our findings differ from those of Price and Dance (8) is that we used a reaction time of <7 min.

Geometry of the post-column reactor system. HPLC systems involving post-column reactors for the detection and the quantification of isoenzymes have already been reported.
(18-21). Vacik and Toren (22), exploring the possibility of using capillary or packed-bed reactors, found that packed-bed reactors demonstrated less band spreading than did coiled capillary reactors for a given residence time but were mechanically unstable. We examined the performances of these two reactor types, the capillary reactor used in our routine method and the packed-bed reactor described in Materials and Methods. Table 1 lists the enzyme activities calculated from the peak areas obtained after the injection of an equal volume of urine filtrate into each system. This experiment yields at least two conclusions:

First, the packed-bed reactor was more efficient than the CTR; although residence time was longer in the CTR we used, higher enzyme activities were obtained by using the packed-bed reactor. When the flow rate of the substrate pumped through these post-column reactors was increased, both the residence time of the reaction mixture in the

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**Fig. 1.** Typical chromatograms of NAG isoenzymes as eluted from column of Mono Q HR 5/5 resin, in urines of three renal-transplant patients with stable grafts.

Other chromatographic conditions are described in text. NAG isoenzyme A activities are 28.5, 26.3, and 25.2 U/mmol of creatinine, respectively, in chromatograms I, II, and III, and isoenzyme B activities are 32.5, 21.8, and 18.0 U/mmol of creatinine, respectively.

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**Fig. 2.** Effect of added NaCl on total NAG activity (NAG A + NAG B), in three different urines of hospital patients without renal impairment

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**Fig. 3.** Effect of the pH of the reaction mixture on total NAG activity (abscissa) urine of a hospital patient without renal impairment

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**Fig. 4.** Effect of the pH of the mobile phase on (A) the pH of the reaction mixture and the isoenzyme activities and (B) the separation of isoenzymes

Left abscissae (A and B): NAG activity
reactor and enzyme activities decreased, as would be expected. With the packed-bed reactor, however, the decrease in enzyme activities did not vary in proportion to the increase of the flow rate. The relatively important enzyme activities we noted at the 1.5 mL/min flow rate suggested that mixing of the substrate and enzyme was better when a greater pressure was involved. Presumably, the increase of pressure could improve the penetration of the reaction mixture in the small capillary tubing, thereby increasing the distance through which a molecule must travel.

Second, with the packed-bed reactor, we observed no enzyme activity when glass particles were used, whether silanized or not; the glass beads had to be previously saturated with albumin. Ellis et al. (9) observed a similar phenomenon and resolved this problem by mixing human albumin with a substrate solution pumped continuously into the packed-bed reactor. To date, these observations have not been explained but may be due to a surface modification of the glass beads by albumin; adsorption of this substance onto the surface of glass particles yielded hydrophilic media that did not denature enzymes or interfere with their retention in the glass bead column. On the other hand, the packed-bed reactor clogs rapidly with usage. Thus we prefer using a coiled tubular reactor, which remains volumetrically stable.

Fig. 5. Effect of substrate concentrations on total NAG activities (NAG A + NAG B) (abscissa) in urines of two different hospital patients.

Table 1. Performances of Two Post-Column Reactors at Various Flow Rates

<table>
<thead>
<tr>
<th>Flow rate, mL/min</th>
<th>Reaction time, s</th>
<th>Enzyme activity*</th>
<th>Reaction time, s</th>
<th>Enzyme activity*</th>
<th>Reaction time, s</th>
<th>Enzyme activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Coiled Teflon tube, 50 mm x 0.5 mm</td>
<td>545</td>
<td>6640</td>
<td>405</td>
<td>4130</td>
<td>315</td>
</tr>
<tr>
<td>1.0</td>
<td>Glass beads saturated with albumin</td>
<td>335</td>
<td>11400</td>
<td>240</td>
<td>6110</td>
<td>230</td>
</tr>
</tbody>
</table>

*Experimental arbitrary units (peak areas)

**No enzyme activity was detected on glass beads (silanized or not silanized) that had not been saturated with albumin.

Precision. Within-run precision (CV), estimated from 15 determinations of the same pretreated sample, was <10% for each isoenzyme (8.5% for NAG A and 9.7% for NAG B).

In summary, the isoenzymes of N-acetyl-β-β-glucosaminidase were easily separated and well resolved by ion-exchange chromatography with the Mono Q anion-exchange resin column. For practical use, combining the HPLC system with a coiled tube enzyme reactor to monitor the column eluate continuously yielded fast and accurate results. The acceptable precision of determinations of isoenzyme A and B activities is attributable to the stability of the chromatographic column and to the reliable reaction in the CTR.

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References


