Automated Determination of Serum Hexosaminidase A by pH Inactivation for Detection of Tay–Sachs Disease Heterozygotes

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We have automated a manual test for detection of heterozygotes of Tay–Sachs disease by assay of hexosaminidase A in serum, based on pH inactivation [Clin. Chim. Acta 43, 417 (1973)]. The same manifold is used both for the total hexosaminidase and pH-inactivation (hexosaminidase B) procedures. Automation expedites mass screening of the Ashkenazi Jewish population for carriers of the Tay–Sachs gene (prevalence rate, 1:30), because 100 or more tests can be performed daily. The mean percentage value and range (±2 SD) of hexosaminidase A for normal adults is 68.6 (58–79) and for carriers is 48.8 (39–59) with the automated pH-inactivation procedure. “Presumed carriers” (<53% hexosaminidase A) and individuals in the uncertain range (53 to 58%) should be retested by using leukocytes, to avoid the effect of certain physical aiments, before being labeled as carriers. The same automated system used for this assay can also be used to detect carriers of at least seven other sphingolipidoses for which artificial fluorogenic substrates are available.

Additional Keyphrases: carrier detection • AutoAnalyzer • fluorometric enzyme assay • automated pH-inactivation • comparison with manual heat-denaturation method • mass screening • sphingolipidoses • lipid storage diseases • normal values

Tay–Sachs disease is an invariably fatal brain lipid-storage disorder, which is inherited as an autosomal recessive trait. The \( \text{G}_{\text{M}2} \)-ganglioside [ceramide-glucose-galactose-(N-acetylneuraminic acid)-N-acetylgalactosamine] accumulates in the central nervous system as a result of inactivity of the enzyme N-acetyl-\( \beta \)-D-hexosaminidase A (Hex A) [2-acetamido-2-deoxy-\( \beta \)-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52] in the biological fluids and tissues of children with Tay–Sachs disease (1), whereas both forms, Hex A and hexosaminidase B (Hex B), of the enzyme are active in normal individuals (2). Obligatory heterozygotes—i.e., carrier-parents—show intermediate Hex A activities between those of normal controls and Tay–Sachs patients (3–5). The ability to detect Tay–Sachs disease carriers (heterozygotes) by mass screening of the high-risk Ashkenazi Jewish segment of a community is based on quantitative measurement of Hex A activity of various biological fluids such as serum (4, 6), leukocytes (3, 5, 6), urine (7), and tears (8) by use of the sensitive, artificial fluorogenic substrate, 4-methylumbelliferyl-\( \beta \)-D-glucosaminide. This substrate reacts at an equal rate with both Hex A and Hex B components of a biological fluid at pH 4.5 to release the highly fluorescent 4-methylumbelliferone, which serves as a measure of the total hexosaminidase activity (9).

For assay of Hex A activity of a biological fluid by manual or semi-automated procedures, three general principles have been used:

(a) Denaturation of Hex A: it is relatively labile to heat as compared to Hex B or the “heat-stable” fraction (4, 10); Hex A is then determined as the difference between the total and the “heat-stable” hexosaminidase activity.

(b) Separation of Hex A from Hex B as a result of differences in their electrophoretic mobility at pH 8.1 in such media as acrylamide gel (3) or cellulose acetate (6, 11). The separated isoenzymes are then immersed in the fluorogenic substrate and the fluorescent bands formed are quantitated with a scanning fluorometer, integrating the areas under each peak.

(c) Hex A activity can also be destroyed by means of pH inactivation, 5-min incubation in pH 2.80 glycine hydrochloride buffer at 37 °C, without appreciably affecting the activity of Hex B (12).
This last procedure is the more readily adaptable to continuous-flow automated (Technicon) systems such as those being used in many clinical chemistry laboratories. We describe here a fully automated system for determination of Hex A activity in serum, in which 100 or more samples can be run daily by a single technician and by which it is feasible to screen large numbers of individuals of the Ashkenazi Jewish community for potential carriers of the Tay–Sachs gene (13).

Materials and Methods

A. Reagents for the pH Inactivation Procedure

1. Sodium citrate buffer, pH 4.5, 0.5 mol/liter. In a volumetric flask, dilute 280 ml of sodium citrate (1.0 mol/liter) and 220 ml of citric acid (1.0 mol/liter) to 1 liter with distilled water. If necessary, adjust to pH 4.5 ± 0.05 and store at 5°C when not in use.

2. Glycine hydrochloride buffer, pH 2.80, 0.5 mol/liter. To 500 ml of glycine solution (1.0 mol/liter) in a liter volumetric flask add 140 ml of HCl (1.0 mol/liter), dilute to the mark with distilled water, and mix. Adjust to pH 2.80 ± 0.02 and store at 5°C when not in use.

3. Fluorogenic substrate. Dissolve 100 mg of 4-methylumbelliferyl acetate and 2-deoxy-D-glucopyranoside (Koch–Light Research Products International, Elk Grove, Ill. 60007) in 100 ml of the sodium citrate buffer. This solution should be prepared just before use.

4. Glycine carbonate buffer, pH 10.0, 0.5 mol/liter. Dissolve 37.6 g of glycine and 53.0 g of anhydrous sodium carbonate in 900 ml of distilled water in a liter volumetric flask. Dilute to the mark with distilled water and mix. Adjust to pH 10.0 and store at 5°C when not in use.

B. Reagents for the Total Hexosaminidase Procedure

1. Sodium citrate–glycine hydrochloride buffer mixture (2:1 by vol). This reagent is a mixture of the first two reagents above. Mix two volumes of the sodium citrate buffer with one volume of the glycine hydrochloride buffer. All other reagents are the same as those described above for the pH-inactivation method.

Note: All reagents should be refrigerated when not in use and 0.5 ml of Brij-35 (Technicon Instruments Corp., Tarrytown, N. Y. 10591) per liter of solution should be added to all reagents used for the AutoAnalyzer procedures.

C. Fluorescent Standards

Stock standard, 4-methylumbelliferyl, 1 mmol/liter. Dissolve 17.6 mg of 4-methylumbelliferyl (Koch–Light) in 100 ml of the glycine–carbonate buffer in a volumetric flask.

Working standards. From the stock standard, prepare solutions containing 200, 150, 100, 75, 50, and 25 nmol/ml, by diluting with the glycine–carbonate buffer.

D. Collection and Dilution of Sera

Venous blood (8–10 ml) from healthy adults was allowed to clot while being refrigerated at 5°C, then centrifuged at 2400 rpm (1450 × g) for 15 min in a refrigerated centrifuge (0°C) within a 2- to 24-h period. The clear serum was stored at −20°C until assayed. The enzyme is stable at 5°C for about two weeks, but for at least six months at −20°C. A large pool of normal sera was divided into 2.0-ml aliquots and stored at −20°C. Some of these normal pooled sera samples were assayed for Hex A activity by previously published manual procedures in which either heat denaturation (4) and pH activation (12) is used. The remaining aliquots were used as known serum controls during every automated analysis run.

Sera with high hexosaminidase activity should be diluted with citrate (6 mmol/liter)–phosphate (10 mmol/liter) buffer (pH 4.5), which is prepared by dissolving 2.68 g of Na2HPO4·7 H2O and 1.15 g of citric acid in 800 ml of distilled water, diluting to 1 liter, mixing, and adjusting the pH to 4.5.

E. Method of Analysis

1. For total hexosaminidase activity. Figure 1 shows the flow diagram of the manifold for the automated system used for both the total hexosaminidase and the pH-inactivation (Hex B) assays. The equipment used is the AutoAnalyzer II system (Technicon) designed for hexosaminidase analysis. It consists of a Sampler IV with a 60 sample/h (1:1) cam, a Proportioning Pump III, a Fluoronephelometer

![Fig. 1. Flow diagram for measurement of N-acetyl-β-D-hexosaminidase B (Hex B) activity by pH inactivation](CLINICAL CHEMISTRY, Vol. 20, No. 5, 1974 539)
with a flowcell (No. 126-B014-02), primary filter (355 nm, No. 7-60) and secondary filter (460 nm, No. 48, plus 426 nm, No. 3-73) and a single-pen AutoAnalyzer Recorder modified for fluorometric measurements.

The reagents used for the total hexosaminidase procedure are those shown in Figure 1, except that instead of the pH 4.5 and pH 2.8 buffer solutions, the buffer mixture (Reagent B1) is pumped through both lines, and the 37 °C bath for the II-B coil is disconnected.

With all reagents pumping, set the fluorometer standard calibration control to 4.00 and adjust the reading on the recorder chart to 10 chart units with the baseline control knob. A 200 nmol/ml standard is aspirated continuously for 10 min and the steady-state value is adjusted to 90 chart units on the recorder chart with the standard calibration control. The reference aperture is set at 2 and the sample aperture at 1.

A series of fluorescent standards (figures are in nanomoles per milliliter) are then placed on the sampler wheel in the following order: 200, 200, 200, water, 200, 150, 100, 75, 50, water, control serum and a series of unknown serum samples, interspersed with control sera. Where available, sera from a child known to have Tay–Sachs disease and from a known carrier—i.e., an obligate heterozygote—should be included with each run. It is unnecessary to run sample blanks. Figure 2 illustrates a set of curves obtained with the automated total hexosaminidase method. Readings obtained for the fluorescent standards are plotted on graph paper, and the values for the unknown sera are calculated from the standard curve in terms of 4-methylumbelliferone (in nanomoles per milliliter) released.

2. pH-inactivation procedure for hexosaminidase B. The flow diagram of the manifold and the reagents used for the pH-inactivation method for Hex B is the same as that shown in Figure 1. Before beginning the run, the 37 °C bath for the II-B coil is reconnected. No washing step is required before changing from the total hexosaminidase procedure to the pH-inactivation method. The reagent baseline is established at 10 chart units and the steady-state value at 90 chart units for the 100 nmol/ml standard exactly as described above for the total hexosaminidase method. Aperture settings are the same. Standards, control, and unknown sera are also run in the same manner as described above, except that the range of the standards is now from 100 to 25 nmol/ml. A set of curves for the pH-inactivation method is illustrated in Figure 3. The values of unknown sera are calculated from a plot of the standard curve in terms of 4-methylumbelliferone (in nmol/ml) released.

After the pH run is completed, the entire system should be washed with NaOH (1 mol/liter) for 5 min, with methylaminopropanol buffer (0.1 mol/liter, pH 10.4; Sigma Chemical Co., St. Louis, Mo. 63178) for 10 min, and with distilled water for 20 min.

Fig. 2. Recorder tracings obtained in experimental run with the automated total hexosaminidase procedure
Sera are designated S; other numbers are nmol/ml

Fig. 3. Recorder tracings in an experimental run with the automated pH inactivation (Hex B) procedure
Sera are designated S; other numbers are nmol/ml

before performing any subsequent runs.

3. Calculations

\[
\text{Hex A (nmol/ml)} = \text{Total Hex} - \text{Hex B}
\]

or

\[
\text{Hex A (%)} = 100 - \left( \frac{\text{Hex B}}{\text{Total Hex}} \times 100 \right)
\]

Results

Although the manifold illustrated in Figure 1 was designed for the pH-inactivation method, the same manifold is used for the total hexosaminidase procedure and, as mentioned, necessitates only the substitution of the mixed buffer solution for the pH 4.5 and pH 2.80 buffers. We have found it to be more
expedient to first assay a series of unknown sera for total hexosaminidase and then to proceed to the pH-inactivation method, with the samples kept in the same sequence, since no washing step is then needed for the changeover. Although a single technician can assay 100 samples in a 7-h working day at a sampling rate of 60 samples per hour, the work output could readily be doubled by purchase of additional automated equipment, so that both the total hexosaminidase and Hex B curves (Figures 2 and 3) are recorded simultaneously with a two-pen recorder.

The reproducibility of the standard curves for both methods is illustrated in Figures 2 and 3 and by the data for 10 consecutive standard runs (Figure 4). As is evident from the curves in Figures 2 and 3, there is a 7% carryover for both the total hexosaminidase and pH-inactivation methods when the highest standard is followed by the lowest one. While such errors can be best eliminated by re-running the samples following those with elevated hexosaminidase values, keeping the unknown sera in the same sequence for both procedures will also serve to reduce the carry-over error to negligible proportions. For example, when the normal control serum was run between two sera with supranormal total hexosaminidase activity—e.g., pregnancy and carrier—sera a slight carryover occurred in both the total hexosaminidase and pH-inactivation runs, but the Hex A value, because it is calculated as a percentage of the total, remains relatively constant, being 60, 59, and 59% for the normal control.

The coefficient of variation for the automated pH-inactivation procedure is 7.6%, as compared to 7.9% for the manual heat-denaturation method. Although such a small difference has little significance, it should be noted that each heat denaturation value is an average of triplicate determinations, whereas the coefficient of variation for the pH-inactivation method was calculated from data for single samples. The manual pH-inactivation procedure (12) has a coefficient of variation of 6.7% for determinations in duplicate.

**Normal Values**

Sera from 170 normal adults were obtained from a population of apparently healthy individuals of both sexes, 17 to 60 years old. Hex A was assayed in these samples by both the manual heat-denaturation method and the automated pH-inactivation method. The results obtained are presented in Table 1 together with data for sera from 27 known carriers and nine Tay-Sachs children. The normal ranges for Hex A (in percent) as determined by these methods (Table 1) compare closely with that found for 22 normal adults by Suzuki et al. (6), who used a cellulose acetate electrophoretic method: 66.0 ± 4.9 SD.

A comparison of the two methods by means of Student's t-test showed no significant difference at the 1% level. A correlation plot comparing the automated pH-inactivation results with those obtained with the heat denaturation method is presented in Figure 5. The regression equation for the relationship is: $y$ (heat-denaturation) = 0.992$x$ (pH-inactivation) – 0.099; the correlation coefficient ($r$) is 0.89, which is significant at the 1% level.

**Discussion**

Although there is practically no statistical overlap between the normal and carrier ranges with the automated pH-inactivation procedure, results between 53 and 58% Hex A are inconclusive (Figure 5) and require careful re-investigation before a person is labeled a carrier. Such sera are re-assayed by both the pH-inactivation and heat-denaturation methods. If

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**Table 1. Serum Hexosaminidase A Activity (in Percent of Total), as Obtained by the Automated pH-Inactivation and by the Manual Heat-Denaturation Methods**

<table>
<thead>
<tr>
<th>Group (men and women)</th>
<th>Automated pH-Inactivation Hex A, %</th>
<th>Manual heat-denaturation Hex A, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Mean ± SD</td>
<td>Range ± 2 SD</td>
</tr>
<tr>
<td>170</td>
<td>68.6 ± 5.2</td>
<td>58–79</td>
</tr>
<tr>
<td>Carriers (Tay-Sachs parents and others)</td>
<td>27</td>
<td>48.8 ± 5.1</td>
</tr>
<tr>
<td>Children with Tay-Sachs disease</td>
<td>9</td>
<td>13.0 ± 6.8</td>
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the initial result is confirmed, then a leukocyte sample is obtained from these individuals as well as from those persons whose results place them unequivocally within the carrier range—i.e., <53% Hex A. Such individuals are placed in the category of “presumed carriers,” because there are a number of physical ailments such as pregnancy, diabetes, and perhaps other unsuspected debilitating disorders that lead to Hex A values within the carrier range (4, 14, 15). In general, leukocyte Hex A values (16) are uninfluenced by such conditions and all “presumed carriers,” which constitute between 3 and 5% of the Ashkenazi Jewish population, should be re-tested by use of both the heat-denaturation (4) and acrylamide gel (3) procedures. A decision is then made as to whether an individual being tested is a “carrier” of the Tay–Sachs gene based on the values obtained for leukocyte Hex A (16).

Whenever possible, it is preferable to have both a husband and wife tested simultaneously, because if only one of them is a carrier they can then be relieved of the anxiety that their mating will result in the birth of a Tay–Sachs child. If both should prove to be carriers, genetic counseling should be instituted without delay, and the parents should be assured that the birth of a Tay–Sachs child can be prevented by monitoring each pregnancy by amniotic fluid analysis (16, 17). It is also advisable at this point to provide genetic counseling to carriers of Tay–Sachs disease and their families, because testing their close relatives facilitates detection of other carriers (18).

In addition to detecting the presence of carriers of the Tay–Sachs gene in the Ashkenazi Jewish population, the automated system described here for determination of total hexosaminidase and Hex B activities may also be useful for detection of carriers of other genetic disorders which afflict the non-Jewish population, such as Sandhoff’s disease (19), in which there is an absence of total hexosaminidase activity. In addition, there are artificial substrates commercially available for at least seven other sphingolipidoses for which the enzyme defects are known, and for which the enzyme activity results in the release of 4-methylumbelliferone (17, 20). Carriers of these genetic disorders could also be detected with the automated system described here, except for appropriate changes in substrates and buffers.

After this paper was submitted for publication, an article entitled “Automated Assay of Hexosaminidases in Serum” by Lowden, J. A., et al. (Clin. Chem. 19, 1345 (1973)) was published. The basis of this automated assay is the denaturation of hexosaminidase A by heating for 5 min at 60 °C. Unlike the procedure presented here, their method does not use 4-methylumbelliferone standards to determine the enzymatic activity of unknown sera in nmol/ml, but calibrates “the instrument with control sera that were first assayed by the manual method.” Their heat-denaturation method cannot be used for determining the hexosaminidase A content of extracts of tissues, leukocytes, cultured fibroblasts, or amniotic cells. However, their reported serum hexosaminidase A values (as a % total activity) of 66.2 ± 5.4 for normal adults and of 44.1 ± 2.6 for carriers are in good agreement with our values (Table 1).

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References