Automated Method for Determination of Bound N-Acetylneuraminic Acid in Serum

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A modified resorcinol method for sialic acid determination has been successfully adapted to the Technicon AutoAnalyzer. The present method requires only 25 μl of serum, and 30 samples can be analyzed per hour. It may be used to measure sialic acid in concentrations ranging from 10 to 100 mg/liter.

The usefulness of determining protein-bound sialic acid in serum is obvious, because a significant increase in various serum sialglycoprotein concentrations (haptoglobin, orosomucoid, α1-antitrypsin, and ceruloplasmin) has been observed in many pathological states (1, 2). We earlier described (3) a fully automated method in which thiobarbituric acid is used as a reagent. This very sensitive method, specific for free sialic acid, has an inconvenience: it requires first a mild acid-hydrolysis step, which is time consuming and complicates the manifold. In this paper, we present a simpler automated method for the quantitative estimation of bound N-acetylneuraminic acid, which is as sensitive as the thiobarbituric acid assay but which is applicable directly to the serum without a previous hydrolysis step. The method is based on a modification of the Svennerholm resorcinol method (4). Spiro (5) has shown that a mild periodate oxidation of free and bound sialic acid increases the chromogenicity of the sialic acid in the Svennerholm assay.

Taking advantage of these observations, Jourdain et al. (6) reported recently a differential method for determining total and bound sialic acid.

With suitable modifications, we were able to automate the periodate resorcinol technique, avoiding the step in which the chromogen is extracted with an organic solvent. The technique described here was successfully used routinely to determine glycosidically-bound sialic acid, but also can be applied to free sialic acid.

Materials and Methods

Apparatus

Proportioning pump, heating bath, and manifold tubings were purchased from Technicon Instruments Corp., Tarrytown, N. Y. 10591. Sampler, colorimeter, and recorder were from Electro-synthèse, Arcueil, Val-de-Marne, 94, France.

Reagents

All chemicals (obtained from Merck, Darmstadt, Germany) were analytical grade.

Periodic acid: Dilute a stock solution of periodic acid (87.2 g/liter) 10-fold with water. Prepare this solution daily. The stock solution should be kept at −20 °C and is stable for at least two months.

Resorcinol reagent: To 100 ml of a resorcinol solution (20 g/liter) add 800 ml of concentrated HCl and 2.5 ml of cupric sulfate (0.1 mol/liter), and adjust the volume to a liter with distilled water. This reagent is stable at 4 °C for several weeks.

Standard Solutions

Stock solution: Dissolve 30 mg of synthetic N-acetylneuraminic acid (Koch-Light, Conbrook Bocks, England) in 10 ml of distilled water. Store this solution at −20 °C in small aliquots.

Working solutions: Dilute 0.1, 0.2, 0.3, 0.4, and 0.5 ml of this stock solution with water to 1 ml, to give standard solutions containing, respectively, 0.30, 0.60, 0.90, 1.2, and 1.5 g of N-acetylneuraminic acid per liter.

In the procedure as modified to determine small concentrations of N-acetylneuraminic acid, the standard solutions were diluted 20-fold.

Procedure

Figure 1 shows the manifold used in the automated method. With the present technique 25 μl of serum is aspirated, oxidized in a double mixing coil, and reacted directly with the resorcinol reagent at 98 °C to give the colored product; its absorbance is measured at 625 nm. Only 12 min is needed for a determination.

A surfactant ("Brij-35," Technicon), 0.5 ml/liter, was added to the water to ensure a clear liquid flowing through the cell.

To improve the bubble pattern, make all connections with Pyrex tubing (1.5 mm, i.d.) and fit pulse suppressors to the sample reagent and air lines. Operate the sampler at 30 samples per hour with a 1:1 wash:sample ratio.

Kinetic studies have shown that at 25 °C with a 40 mmol/liter periodic acid solution a reaction time of 4 min gives maximal color formation.

The concentration of the HCl in the resorcinol reagent was increased to 6 mol/liter, to completely dissolve the serum proteins.

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Results and Discussion

Analytical Variables

**Standard curve:** The relationship between sialic acid concentration and absorbance at 625 nm is linear in the range 0.30 to 1.5 g/liter, which is up to an absorbance of about 0.6 units (Figure 2).

**Precision:** Precision of the method was determined by assaying 20 samples of three different pooled sera (Table 1).

**Carryover:** A concentrated sample (1.50 g of sialic acid per liter) followed by three different less concentrated samples (0.90, 0.60, and 0.30 g/liter) demonstrate the good reproducibility of the method (Figure 3). Mean carryover was 3.5%.

**Accuracy:** Accuracy of the method was tested by adding pure N-acetylenuraminic acid in three different concentrations (0.30, 0.60, and 0.90 g/liter) to a normal serum. The percentage analytically accounted for was never less than 95% of that added.

**Interfering substances:** Several possible interfering substances were studied: hemoglobin, bilirubin, lipids, and the sugars galactose, mannose, and fucose, which are the principal glucidic components found in serum glycoproteins.

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Table 1. Results Obtained for Repeated Analysis of Three Different Pools of Serum

<table>
<thead>
<tr>
<th>Sample conc, g/liter</th>
<th>No. samples</th>
<th>Mean absorbance (625 nm) ± SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.370</td>
<td>20</td>
<td>0.141 ± 0.0022</td>
<td>1.55</td>
</tr>
<tr>
<td>0.753</td>
<td>20</td>
<td>0.289 ± 0.0025</td>
<td>0.86</td>
</tr>
<tr>
<td>1.333</td>
<td>20</td>
<td>0.927 ± 0.0054</td>
<td>1.03</td>
</tr>
</tbody>
</table>

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Fig. 3. Representative AutoAnalyzer recordings

These demonstrated that carryover between the highest and the lowest concentrations used in the standard range is minimal.

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To a pool of serum, hemoglobin and bilirubin were added to final concentrations ranging from 225 to 580 mg/liter and from 75 to 150 mg/liter, respectively. Heparin was added in a concentration of 100 to 600 mg per liter of serum. Galactose, fucose, and mannose were tested at a concentration of 3 mmol/liter. None of these interfered with determination of N-acetylenuraminic acid in serum. To evaluate the influence of lipids, we precipitated the serum lipoproteins with a heparin solution (10 g/liter) according to Burstein and Samaille (7). For normal sera, we saw no decrease in color formation after such separation of the lipoproteins. However, in one pathological serum (total lipids, 11 g/liter), removal of lipids decreased the absorbance by 2.6%. To test the direct influence of lipids and fatty acids on the resorcinol test, cholesterol, cholesterol oleate, cholesterol palmitate, and oleic acid methylester were added in different amounts to a normal serum. We found that all these substances interfered nonspecifically with the assay by causing a turbid solution. As an example, for a concentration of 3 g of cholesterol oleate per liter, which represents the average value for unsaturated lipids and cholesterol in normal serum (9), the increase in absorbance was consistently between 1 and 2%.

Comparison with the Manual Method

To correlate results by the present method with those by known techniques, we used as a reference assay the manual procedure of Aminoff (8). The sera were hydrolyzed in 50 mmol/liter H₂SO₄ at 80 °C for 1 h.

Thirty-four sera from normal individuals were assayed by both methods.

Values obtained with the manual method ranged from 510 to 785 mg/liter (mean ± SD = 658 ± 63). With the automated method, the same sera gave values ranging from 560 to 835 mg/liter (mean ± SD = 682 ± 62).

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Fig. 2. Standard curve for sialic acid with the automated method

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Fig. 1. Diagram of AutoAnalyzer manifold for automated sialic acid determination

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Free Amino Acids in Human Tonsillar Tissue

Yasuyuki Doi and Akikatsu Kataura

Free amino acids in the tonsils of 20 individuals were measured column chromatographically. Those always found in readily detectable amounts included O-phosphoserine, taurine, O-phosphoethanolamine, aspartic acid, hydroxyproline, threonine, serine, glutamic acid, proline, glycine, alanine, α-amino-n-butyric acid, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, γ-amino-butyric acid, lysine, histidine, and arginine. Results were compared for three clinical pathological groups and for four age groups. Some abnormal values may result from the pathological conditions.

Information on the concentrations of the free amino acids in the tonsil should contribute to our understanding of tonsillar pathological physiology.

There have been a few reports on the free amino acids in human tonsils, as measured by paper chromatography (1). This paper reports results of column-chromatographic quantitative and qualitative analysis for 29 amino acids and other ninhydrin-positive compounds in human tonsils.

Materials and Methods

Specimen Preparation

Forty tonsillar specimens were collected from 30 patients who had undergone tonsillectomies in our hospital. Exactly 1.0 g of the tissue was homogenized with a Teflon homogenizer. A slight modification of the method described by Stein and Moore (2) was used to deproteinize the homogenized fluid with 60 ml of picric acid solution (10 g/liter), and the mixture was centrifuged. Picric acid was removed from the supernatant fluid with a small column of Dowex 2 × 10 anion-exchange resin. The effluent and washings were combined and passed through a column of Amberlite IR

References


Additional Keyphrases: lymphatic organ • focal infection • changes with age and disease

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