Improved Automated Determination of Bound
N-Acetylneuraminic Acid in Serum

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The method in which thiobarbituric acid is used for sialic acid determination was adapted to a fully automated procedure, in which the chromagen need not be extracted with an organic solvent. The entire procedure, including hydrolysis, requires 35 min and 25 μl of serum for one analysis, but 30 samples can be analyzed per hour. The procedure, slightly modified, can be used to measure as little as 10 mg of bound sialic acid per liter.

Additional Keyphrases: monitoring sialoglycoprotein changes in serum • "acute-phase reactant" proteins • AutoAnalyzer • thiobarbiturate reagent for sialic acids

The sialic acids have received special attention as normal constituents of various glycoproteins. Among them, certain circulating sialoglycoproteins, called "acute-phase reactant" proteins, increase in concentration in the serum in a number of pathologic states (1, 2). Such changes can be simply followed by determining sialic acids (practically all is N-acetyleneuraminic acid) in sera of patients. There is a good correlation between the concentration of haptoglobin and orosomucoid in serum and measurements of bound sialic acids in inflammatory states (3).

Many methods were developed for determining sialic acids in biological materials. The two most sensitive, one proposed by Aminoff (4) and the other by Warren (5), both involve the use of thiobarbituric acid as a reagent and are relatively specific for free sialic acids.

Two authors adapted these methods to the AutoAnalyzer (Technicon Corp., Tarrytown, N. Y. 10591). Kendall (6), following Aminoff's technique, used in his method a phase separator to extract the butanol-acid phase containing the chromogen from the aqueous phase. Delmotte (7), omitting this step, adapted Warren's procedure to the AutoAnalyzer. For both methods the serum samples have to be hydrolyzed before they are transferred to the AutoAnalyzer, because the thiobarbituric acid assay can only be applied to free sialic acids.

In this paper we present a fully automated procedure based on Aminoff's technique. It includes the acid hydrolysis of the samples and avoids the extraction of the chromogen into an organic solvent.

Material and Methods

Apparatus

A Technicon sample pump and a 98 °C heating bath containing two mixing coils are attached to a colorimeter and a chart recorder (the one we used was purchased from Electro-Synthèse, Arcueil, Val-de-Marne, 94 France).

Reagents

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

Sulfuric acid, 25 mmol/liter, with 0.5 ml of "Brij 35" surfactant (Technicon) added per liter.

Periodic acid, 25 mmol/liter of dilute (62.5 mmol/liter) H₂SO₄.

Sodium arsenite, 20 g/liter of 0.5 molar HCl.

Thiobarbiturate (2-thiobarbituric acid), 0.1 mol/liter of distilled water, adjusted to pH 9.0 with 1 molar NaOH (about 90 ml/liter of solution). The reagent is filtered and kept at 4 °C in a dark bottle.

Standard Solutions

Stock solution: 10 mg of synthetic N-acetyleneuraminic acid (NANA) (Koch-Light, Conbrook Bocks, England) is dissolved in 5 ml of distilled water.

Working solutions: 0.40, 0.60, 0.80, 1.00, 1.20, and 1.40 ml of stock solution is diluted to 2 ml with distilled water to give solutions corresponding, respectively, to 400, 600, 800, 1000, 1200, and 1400 mg of NANA per liter. Stock and working solutions are stored at −20 °C.

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**Method**

*Procedure.* Figure 1 shows the manifold used for the automated thioharbituric assay.

Sample hydrolysis and reaction of the oxidation product of NANA with thioharbituric acid are effected in a single 98 °C bath, which contains two standard Technicon time-delay coils.

The sample is diluted with H₂SO₄ (25 mmol/liter). After passing through the first coil in the heating bath for hydrolysis, it is then cooled in a double mixing coil. Free sialic acid is oxidized in a time-delay coil at 37 °C, and the excess periodate is reduced by sodium arsenite. After thioharbiturate is added, the condensation reaction takes place in the second coil of the heating bath, yielding the final colored reaction product.

Without being cooled, the colored liquid passes immediately through a 15-mm tubular flow cell in the colorimeter and absorbances are read at 550 nm.

All connections are made of Pyrex tubing (1.5 mm i.d.), to improve the bubble pattern. Alternate cups of the sampler are filled with sample and distilled water.

A standard is included in the run after each 10 samples to check that the tracing returns to the baseline. Pulse suppressors are placed on each reagent line and also on air tubing.

At the point where arsenite is added, a precipitate sometimes appears, but it dissolves in the thioharbituric reagent and therefore does not influence the assay. At the end of each run, the entire manifold is first washed with HCl (1 mol/liter), then with distilled water.

*Hydrolysis.* At 80 °C, the highest value was observed for NANA in the serum after 60 min of hydrolysis with 50 millimolar H₂SO₄ (8).

At 100 °C there is a more rapid release of NANA even in 5 millimolar H₂SO₄, as shown by Aminoff (4). With the constant 5-min hydrolysis imposed by the experimental set up, the influence of H₂SO₄ concentration (2.5 to 250 mmol/liter) on the amount of NANA released at 98 °C was studied, a control sample of NANA being treated in the same range of concentration to determine the stability of NANA under these acid conditions (Figure 2).

With H₂SO₄ (25 mmol/liter), the highest values were seen for NANA and corresponded to the amount obtained with the manual method under the conditions already mentioned (H₂SO₄, 50 mmol/liter, 1 h, 80 °C). Kinetic studies have shown that NANA was not measurably degraded in these conditions.

Therefore, we used 25 millimolar sulfuric acid in our method.

*Oxidation by periodic acid.* Oxidation was at 37 °C, based on Aminoff's studies (4). Color formation was maximum after a reaction time of 20 min, but, because only 10% less color is formed in 10 min, we used a 10-min reaction time.

**Results**

With this method, one NANA determination requires 35 min from the time of sampling to the time of recording, and 30 determinations can be run per hour. A good return to the baseline was observed when a water wash was placed between samples.

*Standard curve.* There is linear relationship between absorbance at 550 nm and NANA concentration over the range 0–1.4 g/liter.

*Precision.* Reproducibility of the method was assayed with 20 aliquots of the same pool of serum. A mean value of 811 (SD, ±11.7; CV, 1.45%) mg/liter was obtained.
Carryover. Carryover was checked in replicate by assaying a diluted sample (300 mg/liter) followed by a concentrated sample (1 g/liter) and the diluted sample again. Carryover in this case was less than 5%.

Accuracy. The accuracy of the method was checked by comparing it with the original method of Aminoff. Twenty-five sera from individuals were assayed by both methods.

The equation for the regression curve (Figure 3) is \[ y = 1.05x + 3.07; \] \( r \), the correlation coefficient was 0.955.

Values obtained with the manual method ranged from 550 to 785 mg/liter (mean ± SD = 670 ± 60 mg/liter). Parallel experiments with the automated method gave values ranging from 575 to 810 mg/liter (mean ± SD = 707 ± 55 mg/liter).

The values obtained with both methods are similar to the figures reported in the literature (ref. 3, 670 mg/liter; ref. 10, 667 mg/liter; ref. 11, 636 mg/liter; an exception is ref. 9, 530 mg/liter). However, the automated method constantly shows higher values.

Delmotte (7) suggested that these differences may be attributed to the incomplete extraction of the colored reaction product from the serum by the acid butanol solvent.

Recognizing this possibility, Saifer and Gerstenfeld (10) replaced the extraction step by diluting the chromogen with a water-miscible glycol. But if we determine the NANA content of sera by Aminoff's technique and simultaneously run assays by Saifer's "one-phase" procedure, we always obtain a higher ratio value \( A_{540 \text{ nm}}/A_{322 \text{ nm}} \) for Aminoff's method than for Saifer's method (ca. 25%).

As Warren pointed out (5), this fact indicates that an interfering substance is present in the serum, yielding a chromogen that is not extractable by the acid butanol solvent. However, this interference is negligible in the present method and is completely canceled out when pure glycoproteins are analyzed for their sialic acid content. For instance, we found human haptoglobin and rat orosomucoid to contain, respectively, 5.15% and 15.4% NANA by both methods.

Automated determination of NANA in samples containing 10 to 100 mg/liter. To analyze samples with low concentration of NANA, the sample, air, and waste tubings were substituted by tubing having flow-rates of 0.40, 1.20, and 2 ml/min, respectively. The rest of the manifold was unchanged. Various concentrations of NANA were assayed with the same procedure as described above. Recorded peaks obtained with NANA standard solutions from 10 to 100 mg/liter are shown in Figure 4. A sample volume of 0.25 ml is sufficient for one determination.

References