A Sensitive Autoanalytical Method for Sialic Acids

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A sensitive automated chromatographic method is described for determination of sialic acids. The assay is linear from 1.5 to at least 12 nmoles of N-acetylneuraminic acid, and is not affected by acid, salts, protein, or interfering substances in the sample. The carbohydrate groups of several glycoproteins have been examined.

Automated liquid chromatography for the determination of neutral and amino sugars in complex carbohydrates is a well-established method (1). The method can be used to measure these sugars in the range of nanomoles (2,3). We have now developed a comparable system for the determination of sialic acid to complement the existing methods for neutral and amino sugars. This report describes a highly specific automated chromatographic assay for sialic acid in glycoproteins and other complex carbohydrates.

MATERIALS

Column and resin. A water jacketed column, 250 x 3 (i.d.) mm fitted with nylon column adaptors (6 mm o.d.) was obtained from B/R Glass, Inc. (Pasadena, Md.). Anion exchange resin, Rexyn 201 (Cl-), 200-400 mesh, was obtained from Fisher Scientific Co., Pittsburgh, Pa., and was sized by gravitational sedimentation. The fraction of beads sedimenting between 30 and 60 min (20-40 μm) was selected for chromatography.

Reagents. (A) Sodium periodate, 0.08 M in 3.6 N H₂SO₄. (B) Sodium arsenite, 10% (w/v) in 0.1 N H₂SO₄. (C) Thiobarbituric acid, 1% (w/v) in water, adjusted to pH 9.0 with NaOH. The solution was not stored for more than 1 wk. (D) 1-Butanol containing 10% (v/v) of 12 N HCl.

Instruments. The 8-channel proportionating pump, 95°C oil bath with 80 ft x 2.4 mm. (i.d.) reaction coil, and high pressure gauge were obtained from Technicon, Inc. (Tarrytown, NY). Glass fittings and coils, and tubings for the proportionating pump were supplied by Dyn-
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acon/Acculab (Palisades, NY) or by Technicon, Inc. The following components were also used: Milton Roy high pressure pump (for column elution), Coleman-Hitachi 101 Spectrophotometer, and Heath servo recorder EU-20B with log/linear current module EU-20-28. A Haake circulating water bath was used to maintain both the ion exchange column and the periodate reaction coil at 65°C.

PROCEDURE

Analytical system. The arrangement of analytical components is shown in Fig. 1. The manifold tubings were arranged as shown in Table 1. Acidflex tubings were used to pump butanol-HCl because they were more resistant to this mixture than solvaflex³ tubings. Mixing coils with built-in narrow bore side arms were used to improve the flow characteristics of the system and minimize pulsing. All coils, including the extraction coil were mounted horizontally. Narrow bore manifold tubings were employed as pulse suppressors; a tubing of 0.030 in. (i.d.) was inserted

![Diagram](image)

**Fig. 1.** Arrangement of the analytical components. All mixing coils (MC) are 14 turns and 2.4 mm i.d. Technicon part numbers are: MC-1 (water jacketed), 114-0209-01; MC-2, 116-0103-05; MC-3, 105-0086; MC-4, 116-0103-01. Pulse suppressors (PS) are described in the text. C0 and C4 are Technicon glass fitting designations. An outlet to waste was provided between the column and the proportionating pump.

³ Technicon designation.
TABLE I
ARRANGEMENT OF THE MANIFOLD TUBINGS

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Tubing type</th>
<th>Inner diameter (in.)</th>
<th>Nominal flow ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column effluent</td>
<td>Standard</td>
<td>0.040</td>
<td>0.60</td>
</tr>
<tr>
<td>Air</td>
<td>Standard</td>
<td>0.035</td>
<td>0.42</td>
</tr>
<tr>
<td>Reagent A</td>
<td>Standard</td>
<td>0.010</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent B</td>
<td>Acidflex</td>
<td>0.020</td>
<td>0.16</td>
</tr>
<tr>
<td>Reagent C</td>
<td>Acidflex</td>
<td>0.040</td>
<td>0.60</td>
</tr>
<tr>
<td>Reagent D</td>
<td>Acidflex</td>
<td>0.090</td>
<td>2.03</td>
</tr>
<tr>
<td>Aqueous waste</td>
<td>Acidflex</td>
<td>0.090</td>
<td>2.03</td>
</tr>
<tr>
<td>Organic waste</td>
<td>Acidflex</td>
<td>0.056</td>
<td>0.92</td>
</tr>
</tbody>
</table>

in the exit line from the 95°C oil bath, and a tubing of 0.025 in. (i.d.) was used in the air–butanol waste line. A CO$_3$ glass fitting was used to remove air bubbles, and a C4$_3$ fitting to separate phases. Removal of air bubbles before phase separation promoted cleaner separation, and the larger bore of the water waste arm of the C4$_3$ fitting served to absorb momentary pulses of aqueous phase. The output potentiometer of the spectrophotometer was adjusted for maximum output, and a 250-kohm resistor was inserted in series with the EU-20-28 current input lead (center conductor). A BNC connector was installed in the spectrophotometer readout jack, and the spectrophotometer was run off ground (3-prong to 2-prong adaptor used between the line cord and its receptacle). The range selector of the recorder was adjusted so that full-scale deflection corresponded to either 0.05 or 0.10 absorbance units. In either case, noise was less than 1% of full-scale. Absorbance was measured at 550 nm.4

Preparation and application of sample. Samples were hydrolyzed in 0.05–0.10 N H$_2$SO$_4$ for 1 hr at 80°C (4). An aliquot of the hydrolysate containing 1–15 nmole of sialic acid was applied directly to the column and forced into the column with air pressure (the column had been equilibrated in 10% [v/v] acetic acid, as described below). Sample volumes were usually in the range of 50–100 μl, but as much as 0.6 ml of 0.1 N H$_2$SO$_4$ containing 0.2 m NaCl was applied without affecting elution time or resolution.

Elution. The column was eluted with 10% (v/v) acetic acid, at a flow rate of 0.8 ml/min. Under these conditions, sialic acid had a retention

4 The spectrophotometer and recorder used here could be substituted by any colorimeter/recorder system which has a linear output and a sensitivity of 0.1 absorbance full-scale.
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FIG. 2. Chromatogram of a mixture of colitose (3.6 dideoxy-L-xylohexose, 30 nmole) and N-acetylneuraminic acid (7.2 nmole). Colitose elutes at the column void volume; the apparent retention is due to delay caused by the analytical system.

time on the column of about 20 min, which allowed ample separation between the interfering substances in the void volume and sialic acid (Fig. 2). N-acetylneuraminic acid and N-glycolyneuraminic acid had identical retention times in this system, but the color yield was lower in the case of N-glycolyneuraminic acid (4). Normally, regeneration of the column was not necessary so long as the backpressure remained between 250 and 350 psi. When the backpressure exceeded 350 psi, the column was unpacked, and the resin was washed with 2 N NaOH, reequilibrated, and repacked.

Calculation of results. Peak areas (H × W) were calculated from peak height (H) in chart divisions, and peak width (W) at peak half-height in millimeters. Peak widths were measured with a 6X Edscorp comparator (Edmund Scientific Co., Barrington, NJ). Peak area was proportional to sample size over the range of 1.5–12 nmole (Fig. 3). When an arbitrary constant C, defined as peak area/nmole, was calculated for 10 N-acetylneuraminic acid standards (at varying levels) run on four different days, the standard deviation of the C values was 3.9%. Since aging of the manifold tubings causes some fluctuation in C value, a standard is routinely run for each two or three unknowns.

Analysis of complex carbohydrates. Several glycoproteins have been analyzed. The sialic acid content of α1-acid glycoprotein (orosomucoid) determined by this method was 11.3%; values obtained by manual
methods have been reported in the range 10.8–14.7% (5). The method has been used in the analysis of the carbohydrate groups of two previously uncharacterized glycoproteins, dopamine-β-hydroxylase (6), and Rous sarcoma virus glycoprotein g2 (3). This method was also successfully applied to monitor enzymatic digestion of the last glycoprotein by neuraminidase by direct analysis of aliquots of digestion mixture.

**DISCUSSION**

Although the thiobarbituric acid reaction is reasonably specific for sialic acids, deoxysugars, and other substances capable of producing malonaldehydes and related compounds, are known to cause interference (7). In the course of structural studies on glycoproteins, we have observed interfering substances appearing at the void volume position of the column effluent in the present system. Anion exchange chromatography has been used to purify sialic acids for manual determination (4). The method described here, however, is the first automated assay for sialic acid which combines the resolving power of ion exchange chromatography with the specificity and sensitivity of the thiobarbituric acid reaction.

Existing methods for sialic acid determination require at least 5–10 nmoles (8–11). The automated methods which have been described (10,11) employ sampling devices, and offer considerably less sensitivity when used to monitor the effluent from an ion exchange column. Since
peak height is proportional to sample concentration, the dilution incurred during chromatography leads to an overall loss in sensitivity. Even though the present system includes a fractionation step, it allows the determination of as little as 1 nmol of sialic acid. It is insensitive to relatively large amounts of acid, salt, and protein. Thus, acid hydrolysates or enzymatic digests can be analyzed directly. The time per assay is about 45 min.

The system as described is designed for the structural analysis of complex carbohydrates. Used in conjunction with existing high sensitivity automated methods for neutral sugar (2) and amino sugar analysis (3), it allows the automatic determination of all sugar components of glycoproteins. All systems are capable of measuring as little as 1 nmole, and all use efficient chromatographic methods to purify and identify, as well as quantitate the component sugars.

In other applications, where prior chromatographic separation is not necessary, both the speed and sensitivity could presumably be increased by replacing the column with an automatic sampler. The system is made up of relatively inexpensive and versatile components, and can be adapted to other assays. With the spectrophotometer used in our system (or any other equivalent) the wavelengths can be changed more conveniently than with colorimeters utilizing interference filters. The log/linear recorder used in conjunction with the colorimeter was chosen because of its flexibility in chart speed and its sensitivity.

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REFERENCES