Enhancement of the Yu and Ledeen gas–liquid chromatographic method for sialic acid estimation: use of methane chemical ionization mass fragmentography

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Summary The sialic acid present in erythrocyte ghosts was estimated by methane chemical ionization mass fragmentography of the trimethylsilyl methyl glycosides. The internal standard was 3,4,6-tris-trimethylsilyl-α-phenyl-2-deoxy-2-acetamide-D-glucosaminide, as proposed by Yu and Ledeen (*J. Lipid Res.* 1970. **11:** 506–516). The $[MH-16]^+$ions (m/e 610 for the TMS-methylglycoside of *N*-acetylneuraminic acid and m/e 498 for the internal standard) were used for quantifying nanogram levels of sialic acid in the presence of other contaminating substances. This effectively raises the signal-to-noise ratio for the Yu and Ledeen method by at least two orders of magnitude. The sensitivity and linearity of the method, without use of isotopic carriers, were tested using known quantities of *N*-acetylneuraminic acid. The limit of detection was below 0.4 nanograms (approximately one picomole). The useful range of detection was 10 ng–1 μg, showing a large dynamic range.—**Ashraf, J., D. A. Butterfield, J. Järnefelt, and R. A. Laine.** Enhancement of the Yu and Ledeen gas–liquid chromatographic method for sialic acid estimation: use of methane chemical ionization mass fragmentography. *J. Lipid Res.* 1980. **21:** 1137–1141.

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Sialic acid is present on membrane surfaces as the terminal end of oligosaccharide chains of both glycoproteins and glycolipids (1). The amount of sialic acid present on a cell surface may have some effect on membrane properties, such as cell electrophoretic rate (2), lectin binding, cellular adhesion, and cell morphology (1). A method of estimation of sialic acid levels in crude biological samples without interference from gross contamination with other substances is important in order to understand these and other phenomena more completely.

The most often used methods for estimating sialic acid employ colorimetric (3, 4) and enzymatic (5) procedures. However, Yu and Ledeen (6) have established a sensitive method for sialic acid determination based on gas–liquid chromatography. Recently, Mononen and Kärkkäinen (7) proposed a gas–liquid chromatographic–mass spectrometric (GLC–MS) method, using deuterated sialic acid as an internal standard. A report was recently published by Roboz, Suzuki, and Bekesi (8) using isobutane chemical-ionization GLC–MS to assay sialic acid at low levels.

The CI GLC–MS method described in the current report is essentially a modification of that proposed by Yu and Ledeen (6), but yielding higher sensitivity and selectivity, and it can be utilized for sialic acid determination on whole membranes.

EXPERIMENTAL

Fresh, heparinized human blood was used. *N*-acetylneuraminic acid (Sigma Chemical Co.) was used as a sialic acid standard. Phenyl-*N*-acetyl-α-D-glucosaminide (Sigma Chemical Co.) was used as internal standard, as proposed by Yu and Ledeen (6). Redis-

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; CI, chemical ionization; RBC, red blood cell; TMS, trimethylsilyl.

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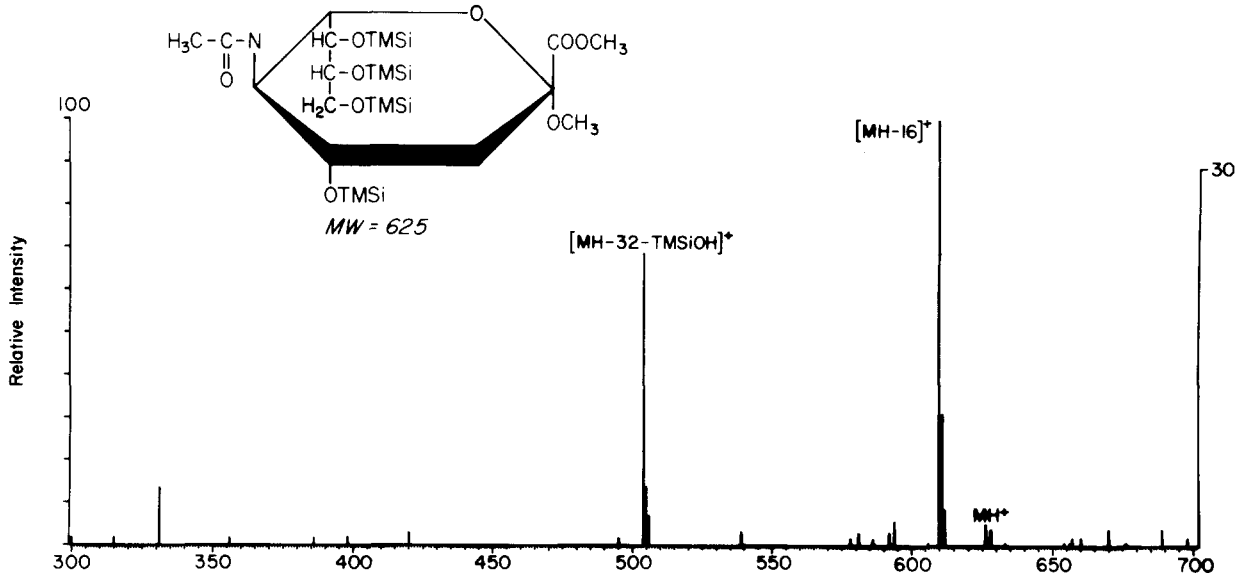

Fig. 1. Methane chemical-ionization (CI)-mass spectrum of the TMS derivative of the methyl ester-methyl glycoside of sialic acid. Instrumental conditions as described in Experimental.

tilled Baker reagent grade methanol was used for methanolysis. Anhydrous methanolic-HCl was prepared by dissolving HCl gas in redistilled methanol. Trimethylsilyl derivatives of the samples were prepared with trimethylchlorosilane–hexamethyldisilazane–pyridine 1:2:5 (9).

Erythrocyte membranes (ghosts) were isolated by two different methods: a) from an aliquot of whole blood (of known hematocrit) on cellulose acetate millipore filters (1 μm) by a method of Schauer, et al. (10) and b) from an aliquot of known volume of washed red blood cells (washed with phosphate-buffered saline). The volume of these cells was measured using tapered graduated centrifuge tubes. The ghosts so prepared were subjected to mild methanolysis (temperature, 90°C; time, 1 hr, concentration of anhydrous methanolic HCl, 0.1 N) followed by filtration or centrifugal separation of undissolved residue. The supernatant was dried and trimethylsilylated as described by Yu and Ledeen (6). About 2 μl were injected into the GLC-column, and the GLC–MS spectra were recorded at 220°C using a Finnigan 3300-6110 mass spectrometer equipped with a column (1.5 m × 2 mm) of 3% OV-1 on 100/120 Supelcoport, with methane as a carrier gas; ionizing electron energy of 150 eV; ion source methane pressure of 1 Torr, and temperature of 60°C. The transfer line was kept at 250°C. The masses monitored were m/e 610 ([MH-16] for sialic acid) and m/e 498 ([MH-16] for internal standard).

RESULTS AND DISCUSSION

The methane chemical ionization mass spectra of sialic acid and the internal standard (3,4,6-tris-trimethylsilyl-α-phenyl-2-deoxy-2-acetamido-D-glucosaminide) are shown in **Fig. 1** and **Fig. 2**, respectively. The ion at m/e 610 was selected for quantitation of the TMS-methylglycoside of *N*-acetylneuraminic acid. The internal standard (TMS-derivative) was followed at m/e 498. These were the [MH-16]ions in each of the above compounds, the higher mass ions being chosen in order to enhance selectivity and minimize serendipidous ions from other compounds.

Quantification was achieved by comparing the sum of the intensities of the m/e 610 across the GLC peak for the TMS-methylglycoside of *N*-acetylneuraminic acid with the sum of intensities of m/e 498 across the GLC peak of the TMS-phenyl-*N*-acetyl-α-D-glucosaminide (internal standard). **Fig. 3** shows the mass fragmentograms of these ions.

The sensitivity and linearity of the method was tested using known amounts of *N*-acetylneuraminic acid. The destruction of this compound under the mild conditions of hydrolysis as described above are almost negligible. A standard curve, generated by graphing the ratio of the peak areas on the ion m/e 610 to m/e 498 as a function of concentration of *N*-acetylneuraminic acid is shown in **Fig. 4.** The response was linear, and the limit of detection was below 0.4 ng (1 pmol). However, for this instrument,

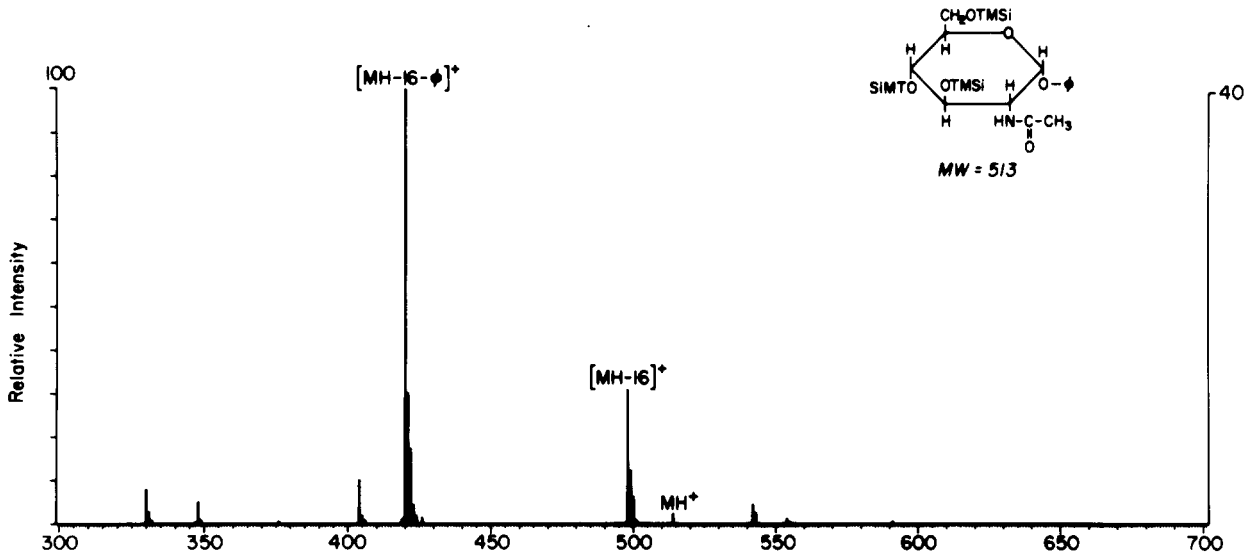

Fig. 2. Methane chemical-ionization (CI)-mass spectrum of the TMS derivative of the internal standard (phenyl-*N*-acetyl-α-D-glucosaminide). Instrumental conditions as described in Experimental.

we recommend a lower analysis curve from 10–100 ng since special tuning of the instrument must be performed to achieve the lower detection limit. In contrast, the limit of detection for the Warren method is 3 μg (10 nmol) and that for the original Yu and Ledeen method is 300 ng (1 nmol).

Several references in the literature (for review, see reference 11) give values ranging from 69–220 μg/ml of sialic acid for packed erythrocytes. For example, Schauer et al. (10) give values ranging from 36.1 to 60.3 μg as uncorrected values per milliliter of packed cells with a mean of 55.0 μg. He corrects these for 20% destruction by his hydrolytic procedure to a range of corrected values from 45.1 to 75.4 μg/ml of cells, with a mean of 69 μg. Our uncorrected values using the Schauer method of isolation range from 33.3 to 41.2 μg/ml with a mean of 38.1 μg/ml of cells (four samples). The Yu and Ledeen analytical method reports 76% yield of sialic acid for pure hematoside and 55% yield for brain tissue. If we assume the 76%,

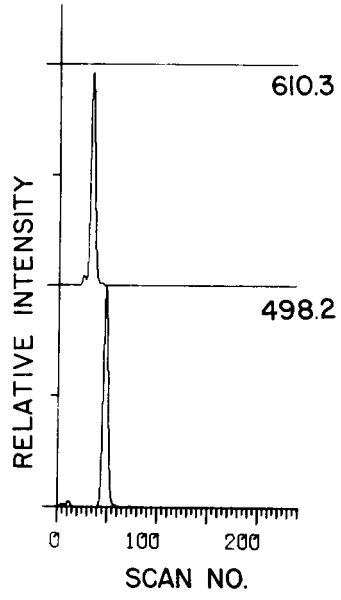

Fig. 3. Mass fragmentograms of sialic acid and internal standard from a preparation of human red blood cells. One μl was injected from a 50 μl sample containing 150 μg of phenyl-*N*-acetyl-α-D-glucosaminide and 170 μg of sialic acid obtained from 0.8 ml cells. Instrumental conditions as described in Experimental.

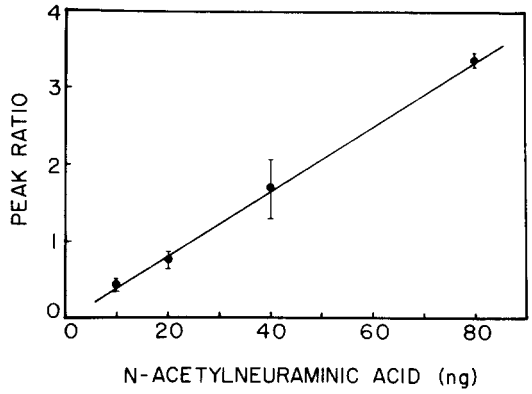

Fig. 4. Standard curve used for the determination of sialic acid in human erythrocyte membranes. The peak ratio is the quotient of the areas under the peaks at m/e 610 to that of m/e 498. Each point represents an average of six to seven determinations. Internal standard, 80 ng.

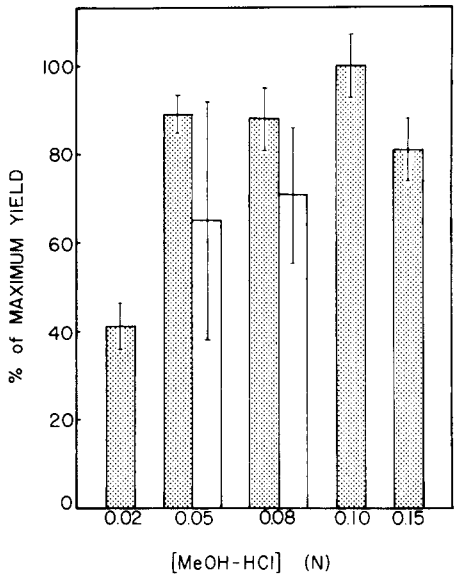

Fig. 5. The yield of *N*-acetylneuraminic acid from red blood cells membranes as a function of concentration of the acid (85°C, 1 hr). ⊠ = anhydrous MeOH-HCl; □ = aqueous MeOH-HCl. In this experiment the maximum yield was set at 100% and others were graphed as percent of the maximum yield.

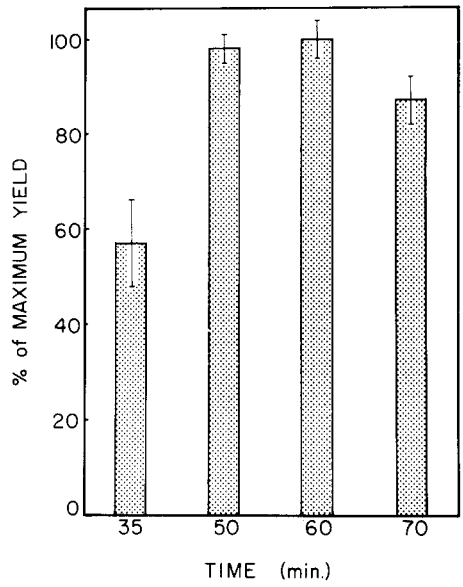

Fig. 7. The yield of *N*-acetylneuraminic acid from red blood cell membranes as a function of time (0.1 N anhydrous MeOH-HCl, 90°C). In this experiment the maximum yield was set at 100% and others were graphed as percent of the maximum yield.

our corrected values would range from 43.8 to 54.2 μg/ml with a mean of 50.1 μg/ml of cells. If we assume the 55% yield, our corrected values would range from 60.5 to 74.9 μg/ml of cells, with a mean of 69.3 μg/ml of cells. It may be more reasonable to assume the latter figure since the sample of whole membranes most resembles the sample of brain tissue. This value agrees very well with the corrected value, 69 μg/ml of cells, of Schauer et al. (10).

We obtained significantly higher values using our modified method which can be summarized as follows: ghosts were isolated by centrifugation from washed erythrocytes of known volume, sialic acid was hydrolyzed under an optimized set of conditions (0.1 N anhydrous MeOH-HCl, 1 hr, 90°C, determined by a series of experiments as illustrated in **Figs. 5–7**). We find that results from this modified preparation yield much higher values of sialic acid than those yielded by any previous method attempted by us. By this improved method our uncorrected values range from 173–204 μg/ml with a mean of 186 μg/ml of packed erythrocytes. If the values were corrected for the 55% yield of sialic acid in the Yu and Ledeen procedure, the values are much higher: 338 μg/ml of packed erythrocytes. The reproducibility of the analysis on a single erythrocyte preparation using anhydrous MeOH-HCl is indicated by the standard deviation bars in Figs. 5–7. Typically, these standard deviations were always less than 10%. The biological variabilities from six individuals fell within this experimental error. We can speculate that the increased values for sialic acid using this method of isolation rather than the method the Schauer et al. (10) may arise because of losses through the cellulose acetate filter or absorption of substances to the filter in an aggregated form resistant to solubilization in the acid.

Sialic acid is determined by the use of methane chemical ionization mass fragmentography using the

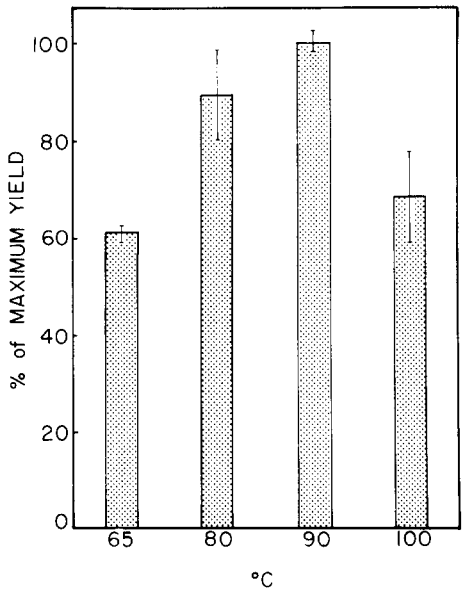

Fig. 6. The yield of *N*-acetylneuraminic acid from red blood cell membranes as a function of temperature (0.1 N anhydrous MeOH-HCl, 1 hr). In this experiment the maximum yield was set at 100% and others were graphed as percent of the maximum yield.

Yu and Ledeen method for preparation of the sample with modifications as described above (6). Since chemical ionization yields a simple fragmentation pattern with a majority of the ion current represented in a few high mass ions, the method is highly specific. The [MH-16]ion, which is very intense, can be used for detection of picomolar levels. Sialic acid can be estimated at these levels without the need for isotopic carriers, even from preparation of whole membranes, as shown by an example with human erythrocyte ghosts. The standard curve recently reported using isobutane-CI-MS (8) ranges from 25–200 ng. The limit of detectability for the method using deuterated carriers is one ng (3.3 pmol) (7).

The specific and sensitive method of determination of sialic acid developed in this work can be utilized to study various pathological states where evidence of a generalized membrane defect has been gained (12, 13).

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