QUANTITATIVE DETERMINATION OF NEURAMINIC ACID BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY

Ilkka MONONEN and Jorma KÄRKKÄINEN

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17, Finland

Received 11 August 1975

1. Introduction

Neuraminic acid is widely distributed in nature, occurring generally as a component of the oligosaccharide units of glycoproteins, gangliosides and of the milk and urinary oligosaccharides. Increased attention has recently been paid to its presence in the membranes of normal and malignant cell surfaces.

Several methods have been developed for the determination of neuraminic acid; most of them are colorimetric [1–3]. Gas chromatographic [4,5], radioisotopic [6] and enzymatic [7] methods have also been described. These methods, however, require relatively pure samples and estimation of amounts under 1 μg from samples of biological origin is often difficult.

In the present work neuraminic acid is determined with gas chromatography-mass spectrometry using the multiple-ion-detection technique. The compound is liberated by methanolysis, re-*N*-acetylated and trimethylsilylated before gas chromatographic analysis. A derivative labelled with deuterium in the methyl groups is used as an internal standard. For quantitation by mass fragmentography two ions (m/e 298 and m/e 420) are recorded, which makes the method highly specific. The method allows an accurate determination of neuraminic acid from 1 ng (3.3 pmol) up to micrograms. As far as the authors are aware, no similar method for the determination of a monosaccharide has previously been described.

The method was applied to the analysis of neuraminic acid in a crude protein fraction from rat brain.

2. Experimental

N-Acetylneuraminic acid was purchased from the Sigma Chemical Company, St. Louis, Mo., and tetradeuteromethanol from E. Merck, Darmstadt, BRD. All other chemicals used were analytical grade commercial products.

Samples for the assay procedure were prepared as described by Bhatti et al. [4]. The sample was methanolyzed in 0.5 ml of 0.5 N methanolic HCl containing 1.5% H_2O at 85°C for 18 h. After neutralization with silver carbonate (200 mg) the sample was re-*N*-acetylated with 60 μl acetic anhydride for 6 h. The precipitate was washed twice with methanol, the solvent evaporated, and the dry sample was trimethylsilylated with trimethylchlorsilane-hexamethyldisilazane-pyridine (1:2:10, v/v/v).

The internal standard was prepared from *N*-acetylneuraminic acid in an identical manner but using trideuteromethanol instead of methanol and was added to the sample after the re-*N*-acetylation step.

The crude protein fraction from rat brain [8] was prepared as follows: the whole brain of a 3-month old male Albino Wistar rat was homogenized in 20 vol chloroform-methanol (2:1, v/v) at 4°C. After centrifugation (20 000 *g*, 30 min) the precipitate was homogenized in 10 vol chloroform-methanol (1:2, v/v) and centrifuged as before. The lipid-free precipitate was used for neuraminic acid analysis.

Mass spectra were recorded with a Varian MAT CH-7 instrument equipped with a Varian Aerograph 1700 gas chromatograph and a Spectro System 100

North-Holland Publishing Company – Amsterdam

S computer system. Glass columns (2 m X 2 mm, i.d.), filled with 2.2% SE-30 on Gas Chrom Q (100–120 mesh) from Applied Science Laboratories (State Collage, Pa.) were used at temperatures 190–220°C. The ionization voltage was 70 eV.

Ion intensities were recorded with a Statos 1 recorder using a time constant of 0.1 sec and repetitive scanning over 10 ions (3 sec per scan). The amount of neuraminic acid was calculated by comparing the sum of intensities under a gas chromatographic peak with that of the corresponding ion in the spectrum of the internal standard.

3. Results and discussion

The mass spectra of the trimethylsilyl ethers of the methyl ester methyl glycoside and of the trideuteromethyl ester trideuteromethyl glycoside of *N*-acetylneuraminic acid are shown in fig.1. For the assay procedure two ions (m/e 298 and m/e 420) were selected. The corresponding ions in the mass spectrum of the deuterium labelled internal standard were m/e 301 and m/e 426. The structures of these fragments have been studied by Kamerling et al. [9] and are shown in fig.1.

The sensitivity and linearity of the method were tested using known amounts of *N*-acetylneuraminic acid (1–1200 ng) with three dilutions of internal standard (10, 100 and 1000 ng). The ratio of the peak areas of ions m/e 298 to m/e 301 and m/e 420 to m/e 426 are shown in fig.2. The response was linear using both ions and the ratio of the internal standard to the sample can be varied over a wide range without affecting linearity. The limit of detection was below 1 ng with the more intense ion m/e

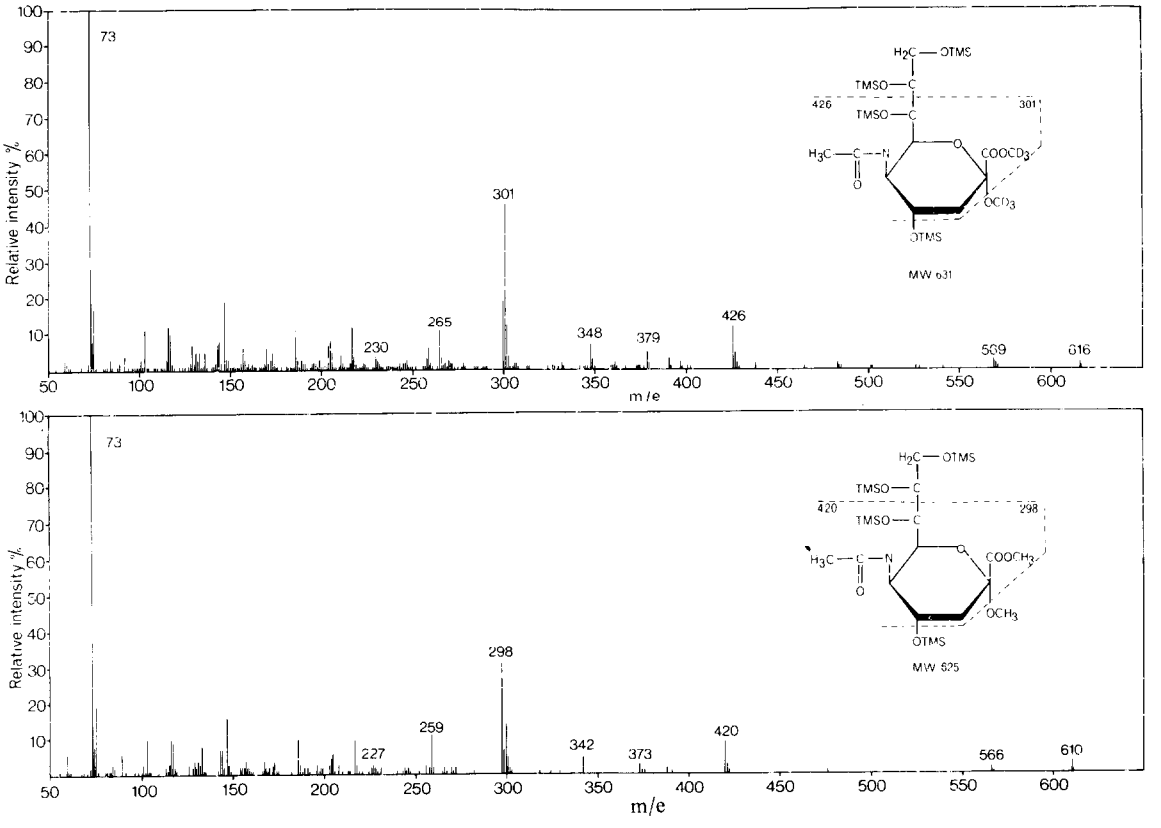

Fig.1. Mass spectra of trimethylsilyl derivatives of the methyl ester methyl glycoside (below) and of the trideuteromethyl ester trideuteromethyl glycoside (above) of *N*-acetyl-neuraminic acid.

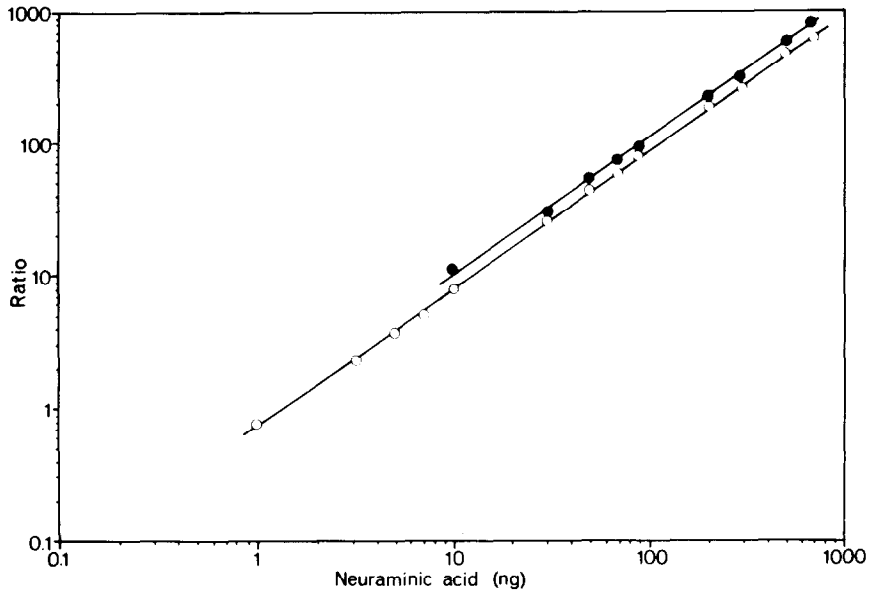

Fig.2. Standard curves for determination of neuraminic acid. Peak area ratios: m/e 298 to m/e 301 (open circles), m/e 420 to 426 (closed circles). Internal standards 10, 100 and 1000 ng. Each point represents the average of 6-8 determinations.

298. The reproducibility of the method was essentially the same for samples greater than 10 ng (standard deviation of the mean was ± 2% for replicate samples). The background interference (equal to approx. 1 ng of neuraminic acid) was constant during the elution of the peak and could easily be subtracted. This interference, which mainly comes from the column and silylation reagents, can probably be further decreased. The use of continuous ion monitoring, instead of repetitive scanning, should also procedure a considerable increase in sensitivity.

To test the applicability of the method to a crude biological preparation, 4 mg of the lipid-free protein fraction of rat brain was subjected to methanolysis and dilutions of the methanolyzate were assayed for neuraminic acid (table 1). There was practically no interference from other substances and the sensitivity of the assay was the same as with pure neuraminic acid. Therefore, in principle, it is possible to determine neuraminic acid from 10 μg of wet brain tissue.

The absolute values do not necessarily represent the true amount of neuraminic acid in the fraction,

Table 1
Neuraminic acid content of a crude protein fraction of rat brain

Protein fraction (μg dry weight)	Internal standard (ng)	Neuraminic acid (ng) m/e 298/301	m/e 420/426
300	1000	820	820
100	500	280	290
30	100	82	86
10	50	29	30
3	10	8.3	
1	7	2.7	

since the degree of liberation of neuraminic acid from tissues by methanolysis has not been adequately studied. The result obtained (0.90 μmol/100 mg protein) compares, however, well with earlier values (0.95 μmol/100 mg) [8] obtained with gas chromatography after more extensive purification.

A major advantage of the mass spectrometric method, besides its sensitivity, is its specificity. This specificity arises from the use of an internal standard of deuterated neuraminic acid to obtain an accurate retention time and the possibility of using several ions for quantitation.

Due to the selectivity of the detection the analysis time can be shortened to 3–4 min which further increases the usefulness of the method.

In the present work, methanolysis was applied directly to a tissue sample, but other methods of liberation could, and probably should, be used to degrade membranes and glycoproteins as a preliminary step. At present the uncertainty in the pretreatment of samples for this of other carbohydrate assays is far greater than those of the final analytical method itself.

Acknowledgements

We thank Professor Johan Järnefelt for valuable advice. The skillful technical assistance of Mrs Maire Ojala and Mrs Kristiina Heinonen is gratefully acknowledged. This work was supported by the Sigrid Jusélius Foundation and the National Research Council for Medical Sciences, Finland.

References

[1] Aminoff, D. (1961) Biochem. J. 81, 384–392.
[2] Warren, L. (1959) J. Biol. Chem. 234, 1971–1975.
[3] Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611.
[4] Bhatti, T., Chambers, R. E. and Clamp, J. R. (1970) Biochim. Biophys. Acta 222, 339–347.
[5] Yu, R. K. and Ledeen, R. W. (1970) J. Lip. Res. 11, 506–516.
[6] McLean, C., Werner, D. A. and Aminoff, D. (1973) Analyt. Chem. 55, 72–84.
[7] Brunetti, P., Swanson, A. and Roseman, S. (1963) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds) Vol. VI, pp. 465–473, Academic Press, New York.
[8] Krusius, T., Finne, J., Kärkkäinen, J. and Järnefelt, J. (1974) Biochim. Biophys. Acta 365, 80–92. 80–92.
[9] Kamerling, J. P., Vliegenthart, J. F. G. and Vink, J. (1974) Carbohyd. Res. 33, 297–306.