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ANALYSIS OF SIALIC ACIDS BY GAS CHROMATOGRAPHY OF THE MANNOSAMINE DERIVATIVES RELEASED BY THE ACTION OF N-ACETYLNEURAMINATE LYASE

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SUMMARY

A convenient method for the analysis of sialic acids is proposed, which is based on their dissociation into pyruvate and N-acylmannosamines by the action of N-acetylneuraminate lyase, followed by gas-chromatographic analysis of the latter products as trimethylsilylated diethyl dithioacetals. Conjugated sialic acids should be freed with neuraminidase before being subjected to the action of the lyase, but these sequential enzymic reactions may be performed in one pot. N-Acetyl-, N-glycolyl- and N,O-diacetylneuraminic acids gave the corresponding mannosamines, and the dithioacetal derivatives of these mannosamines were well separated on a column of silicone OV-1. Quantitation of this enzymic and gas chromatographic method indicated that the error and coefficient of variation for free N-acetylneuraminic acid were 1.1% and 2.5%, respectively, for ten determinations at the 100 nmol level. The values for conjugated N-acetylneuraminic acid in N-acetylneuramin-lactose were 2.9% and 5.9%, respectively. This method was applied to the analysis of sialic acids in some biological samples, and the results were compared with those obtained by the conventional colorimetric method. Preliminary data on urinary sialic acids indicated that cancerous patients gave significantly higher levels of urinary N-acetylneuraminic acid than normal subjects.

INTRODUCTION

Sialic acids occur widely in animals and bacteria as glycosidic components

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of various glycoconjugates [1]. N-Acetyl- and N-glycolylneuraminic acids are the most abundant sialic acids, and the occurrence of isomeric N,O-diacetylneuraminic acids in some tissues has been also reported [2]. Although several colorimetric methods [3, 4] are available for the determination of sialic acids, they are not very selective and the problem of interference is serious in applying them to biological samples, especially serum and urine. The recent enzymic method, based on the measurement of pyruvate released by the action of N-acetylneuraminate lyase (N-acetylneuraminate pyruvate lyase, EC 4.1.3.3) [5] has partially solved this problem, but it can only determine total sialic acids, because all sialic acids commonly produce pyruvate. In contrast with this, gas chromatographic analysis of the other products, namely N-acylmannosamines, by the trimethylsilylated dithioacetal method [6] allows good separation of their derivatives, and hence permits simultaneous analysis of individual sialic acid homologues without interference.

This paper is concerned with the optimization of this enzymic and gas chromatographic method. It also presents some applications of this method to various biological samples.

EXPERIMENTAL

Materials

Clostridium perfringens neuraminidase (mucopolysaccharide N-acetylneuraminidase, EC 3.2.1.18, type IV) and jack bean urease (type C-3) were obtained from Sigma (St. Louis, MO, U.S.A.). *Escherichia coli* N-acetylneuraminate lyase was purchased from Nakarai (Kyoto, Japan). The samples of N-acetyl- and N-glycolylneuraminic acids were from Nakarai, whereas those of N-acetylneuraminlactose, bovine submaxillary mucin (type 1), fetal calf serum fetuin (type III), human serum transferrin and human serum acid glycoprotein were from Sigma. Ethanethiol and chlorotrimethylsilane were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid and hexamethyldisilazane were from Wako Pure Chemicals (Osaka, Japan). All other chemicals, solvents and carbohydrate samples were of the highest grade commercially available.

Urine samples of normal subjects were collected in the early morning, before breakfast, from volunteers of both sexes and various ages ranging from twenties to fifties. Urine samples from cancerous patients were obtained in the same manner. All cancers had not been metastasized and all patients were not at the mortal stage. A 500-μl portion of each urine sample was submitted to sialic acid analysis by procedure 3 (see below). Urinary creatinine was assayed with alkaline picrate by the method of Bonsnes and Taussky [7].

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector. For the analysis of mannosamine derivatives by procedures 1 and 2 (see below), a glass column (1 m × 3 mm I.D.) packed with Chromosorb W AW DMCS (80—100 mesh) coated with 2% silicone OV-1 was used at 190°C, but for the analysis by procedure 3 a SCOT capillary column (50 m × 0.28 mm I.D.) coated with sili-

cone SF-96 was used at 225° C. The flow-rates of the carrier (nitrogen for both columns) were regulated at 50 and 1 ml/min, respectively. Peaks were integrated by a Shimadzu E1A Chromatopak integrator. Gas chromatography-mass spectrometry was carried out on a Hitachi M-70 spectrometer by using the silicone OV-1 column under the same conditions as described above. The ionization potential was 70 eV.

Procedure for the analysis of conjugated sialic acids in glycoprotein preparations (procedure 1)

Dissolve a sample (0.5—1 mg) of a glycoprotein preparation in water (500 μl), and mix the solution with 0.2 *M* phosphate buffer (pH 7.0, 200 μl). Add aqueous solutions of neuraminidase (0.1 U per 50 μl) and N-acetylneuraminate lyase (0.3 U per 60 μl), and incubate the mixture for 1 h at 37° C. Heat the mixture for 1 min at 100° C to inactivate the enzymes, and introduce it onto a column containing Amberlite CG-120 (H^+, 1 ml) and CG-400 (CH_3COO^-, 1 ml). Wash the column with water (30 ml), and evaporate the combined eluate and the washing fluids to dryness under reduced pressure below 40° C. Dissolve the residue in a 1×10^{-3} *M* aqueous solution (100 μl) of 3-O-methylglucose (internal standard), and transfer the solution to a small glass tube (5 cm × 5 mm I.D.) with a small volume of water washings. Evaporate the solution to dryness under reduced pressure by placing the tube in a desiccator containing sodium hydroxide. Dissolve the residue in a 2:1 (v/v) mixture (20 μl) of ethanethiol and trifluoroacetic acid, and keep the solution for 10 min at 25° C. Add pyridine (50 μl), hexamethyldisilazane (100 μl) and chlorotrimethylsilane (50 μl), and incubate the mixture for 30 min at 50° C. Centrifuge the mixture, and inject a 1-μl sample of the supernatant into the OV-1 column. Estimate the amounts of mannosamine derivatives from the relative peak responses to that of the internal standard. The amounts of mannosamine derivatives are equivalent to those of sialic acids.

N-Acetylneuraminic acid in N-acetylneuraminlactose was also determined by this procedure.

Procedure for the analysis of free sialic acids (procedure 2)

Free sialic acids were analyzed by the same procedure as procedure 1, except that the neuraminidase was omitted in the enzyme reaction.

Procedure for the analysis of urinary sialic acids (procedure 3)

Add 0.2 *M* phosphate buffer (pH 7.0, 200 μl) and aqueous solutions of neuraminidase (0.1 U per 50 μl), N-acetylneuraminate lyase (0.3 U per 60 μl) and urease (1 U per 100 μl) to a urine sample (500 μl), and incubate the mixture for 1 h at 37° C. Treat the reaction mixture as described for procedure 1, and analyze sialic acids using the SF-96 capillary column.

RESULTS AND DISCUSSION

Optimization

Free sialic acids were readily split into pyruvate and N-acylmannosamines by the action of N-acetylneuraminate lyase, but conjugated sialic acids were

resistent to this enzyme. However, the latter may be hydrolyzed with neuraminidase, and free sialic acids liberated were exposed to the action of the lyase. Because the optimum pH range (5.0—8.0) of neuraminidase was partially superimposed on that (6.5—8.5) of the lyase, proper choice of the common pH value, preferably 7.0, permitted concerted action of these two enzymes, making one-pot reaction possible. Both enzyme reactions were rapid, and almost complete in 1 h, when 0.1 and 0.3 U, respectively, were used for the 100 nmol amount of substrate. N-Acylmannosamines, formed by a combination of neuraminidase and the lyase (procedure 1), were quantitatively derivatized to their trimethylsilylated diethyl dithioacetals according to the procedure described in our previous paper [6], and the derivatives were analyzed by gas chromatography. From the data obtained, the total amount of free and bound sialic acids could be estimated for each sialic acid species. Schematic expression of these series of enzymic and chemical reations is shown in Fig. 1.

When neuraminidase was omitted from the above system (procedure 2), only free sialic acids could be determined.

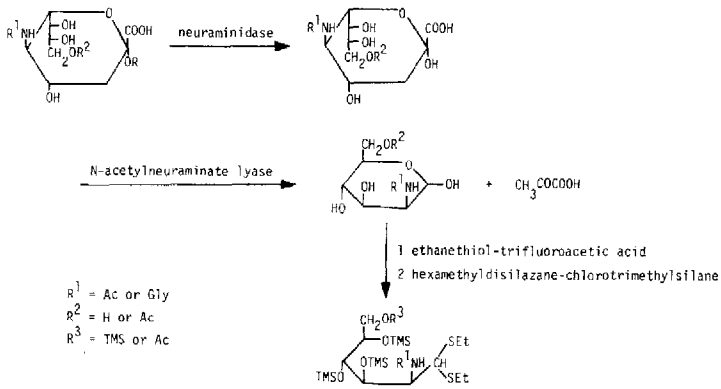

Fig. 1. Scheme of the enzymic and chemical reactions described.

Accuracy and precision

The recovery and coefficient of variation of the determination of free N-acetylneuraminic acid by procedure 2 were 101.1 and 2.5%, respectively, for ten determinations. For the determination of conjugated N-acetylneuramic acid in N-acetylneuraminlactose by procedure 1, the respective values were 97.1 and 5.9%. These data indicate that the present method is accurate and reproducible.

Analysis of sialic acids in glycoprotein preparations

Fig. 2 shows the results of gas chromatography—mass spectrometry of the products obtained by these sequential reactions on sialic acids in commercial bovine submaxillary mucin. The gas chromatogram shows the presence of three peaks (peaks 2, 3 and 4) of N-acylmannosamines, together with a peak (peak 1) of 3-O-methylglucose (internal standard). The retention times of peaks 2 and 4 were identical with those arising from the products obtained by treating N-acetyl- and N-glycolyl neuraminic acids, respectively, by procedure 1. All the

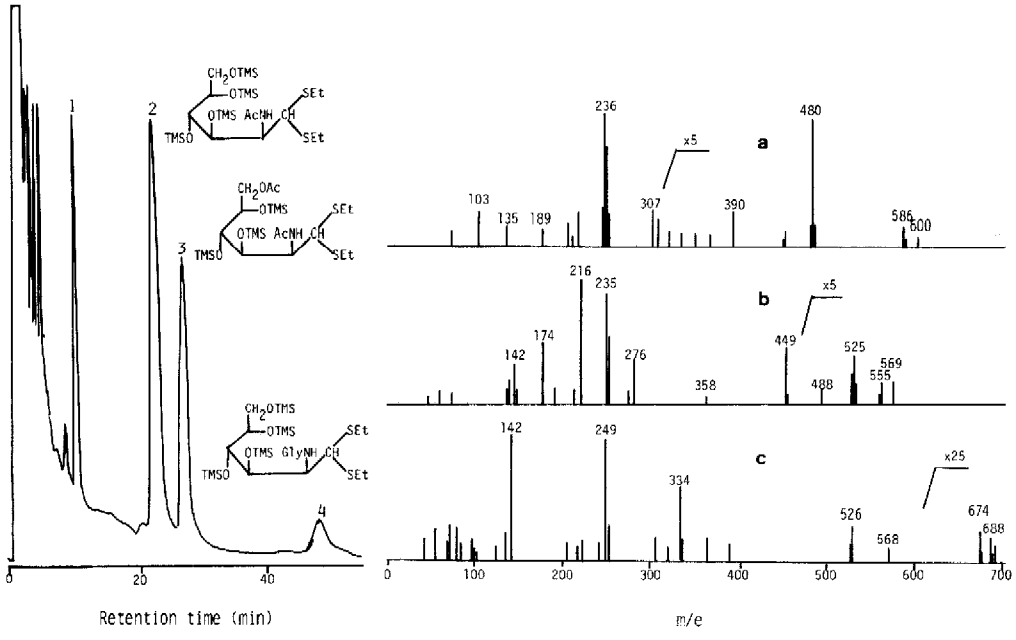

Fig. 2. Gas chromatography—mass spectrometry of the derivatives of N-acylmannosamines derived from the sialic acids in bovine submaxillary mucin by procedure 1. Assignment of gas chromatographic peaks: 1 = 3-O-methylglucose (internal standard); 2 = N-acetylmannosamine; 3 = N,O-diacetylmannosamine; 4 = N-glycolylmannosamine. The mass spectra (a), (b) and (c) represent those of the compounds of peaks 2, 3 and 4, respectively.

mass spectra of peaks 2, 3 and 4 gave no molecular ions, but they all had pairs of fragment ions assignable to $M^+ - CH_2CH_3$ and $M^+ - CH_3$ (586 and 600, respectively, for peak 2; 556 and 570, respectively, for peak 3; 674 and 688, respectively, for peak 4). The molecular weights of the compounds of peaks 2, 3 and 4, as estimated on the basis of these fragmentations, are 615, 585 and 703, respectively. Thus, the compounds giving peaks 2, 3 and 4 were identified as the trimethylsilylated diethyl dithioacetals of N-acetyl-, N,O-diacetyl- and N-glycolylmannosamines, respectively. Regarding the structure of the compound of peak 3, an additional evidence was provided; i.e. its mass spectrum gave fragment ions at *m/e* 175 and 277, assignable to $^5C^+HOTMS—^6CH_2OAc$ and $^4C^+HOTMS—^5CHOTMS—^6CH_2OAc$, respectively. These results suggest that the compound giving peak 3 has an O-acetyl group at C-6, together with an N-acetyl group, in the mannosamine residue, and accordingly the parent sialic acid is N-acetyl-9-O-acetylneuraminic acid. Although the presence of isomeric N,O-diacetylneuraminic acids in bovine submaxillary mucin has been reported in the literature [8], the commercial sample used in this work gave only one peak for diacetylmannosamine.

Table I summarizes the amounts of individual sialic acids in several commercial glycoprotein preparations, as estimated by procedure 1. It also gives the amounts of total sialic acids, as obtained by the conventional colorimetric method based on the reaction with periodate and barbiturate [3]. It is observed that fetal calf serum fetuin and human serum transferrin contained only N-acetylneuraminic acid and its amounts estimated by the present method

TABLE I

DETERMINATION OF SIALIC ACIDS IN VARIOUS GLYCOPROTEIN PREPARATIONS

Glycoprotein preparation	Amount of sialic acid (mg/g)			
	Present method			Colorimetric method [3]
	N-Acetyl-neuraminic acid	N,O-Diacetyl-neuraminic acid	N-Glycolyl-neuraminic acid	Total sialic acids
Fetal calf serum fetuin	53.3	0.0	0.0	53.1
Human serum transferrin	14.7	0.0	0.0	14.6
Human serum acid glycoprotein	105	0.0	0.0	80.0
Bovine submaxillary mucin	11.7	5.9	6.7	25.1

are in good agreement with those obtained by the colorimetric method. Human serum acid glycoprotein also gave only N-acetylneuraminic acid, but its amount exceeded that of the total sialic acids obtained by the colorimetric method. In this case the latter method should have underestimated the sialic acid due to interference by the accompanying protein. The amount of N-acetylneuraminic acid in bovine submaxillary mucin was 47% of that of total sialic acids. The amounts of N,O-diacetyl- and N-glycolylneuraminic acids were approximately one half of that of N-acetylneuraminic acid. The sum of the amounts of individual sialic acids agreed well with the amount of total sialic acids estimated by the colorimetric method. In estimating the amount of N,O-diacetylneuraminic acid the molar response was arbitrarily assumed to be the same as that of N-acetylneuraminic acid, because no authentic sample was available. For the estimation of the amount of N-glycolylneuraminic acid, the relative response of peak 4 to peak 2 was referred to that obtained by applying these sequential reactions on an equimolar mixture of N-acetyl- and N-glycolylneuraminic acids. The reference value was 0.61.

Analysis of urinary sialic acids

Application of procedure 1 to the analysis of urinary sialic acids met difficulty due to interference of the derivatization of N-acylmannosamines to trimethylsilylated diethyl dithioacetals and poor resolution of the gas chromatographic peaks from those of accompanying substances. Since the problem of interference is mainly due to large amounts of urea, this compound was eliminated by adding jack bean urease to the enzyme system of procedure 1. Under these conditions urea was decomposed nearly quantitatively to ammonia and carbon dioxide. On the other hand, the poor resolution of peaks was improved by using a SCOT capillary column coated with silicone SF-96. Based on these results a modified procedure (procedure 3) was devised for the analysis of urinary sialic acids. Fig. 3 shows an example of chromatograms obtained for normal urine samples by this procedure. Peak 6 is assignable to N-acetylmannosamine, but no peaks are detected for N,O-diacetyl- and N-glycolylneuraminic acid. Peaks 1, 2, 4 and 5 are of the derivatives of urinary aldoses, and peak 3 is assigned to 3-O-methylglucose (internal standard).

Table II presents some preliminary data on the urinary N-acetylneuraminic acid content of normal subjects and cancerous patients. The normal level

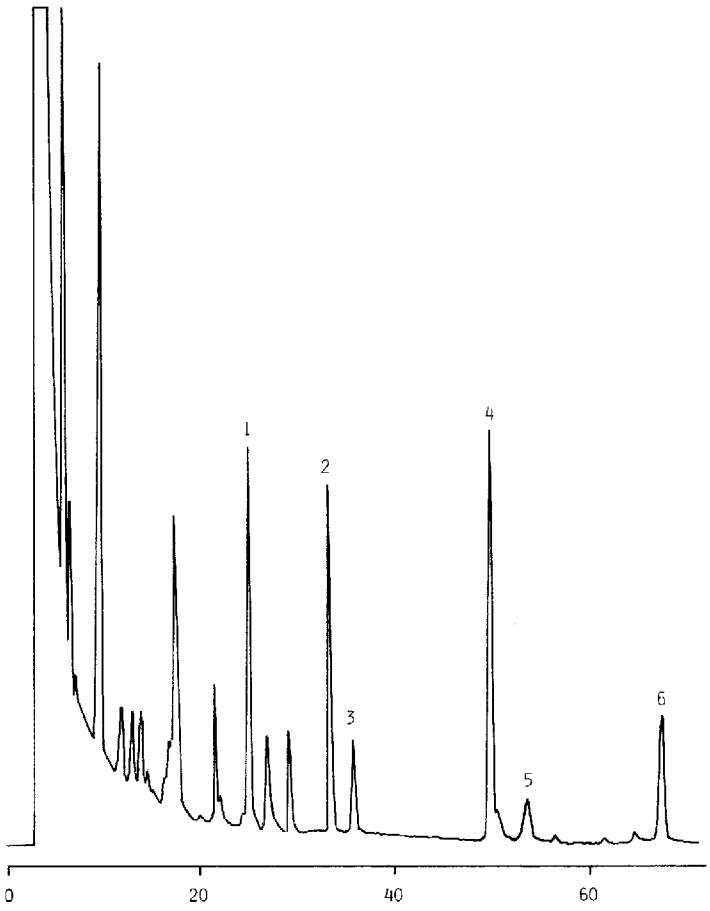

Fig. 3. Gas chromatogram of the derivatives of urinary carbohydrates, obtained by the enzyme reaction of a urine sample from a normal subject, followed by derivatization of the products to trimethylsilylated diethyl dithioacetals according to procedure 3. Peak assignment: 1 = xylose; 2 = fucose; 3 = 3-O-methylglucose (internal standard); 4 = glucose; 5 = galactose; 6 = N-acetylmannosamine.

obtained for eighteen subjects was 21.2 (mean, m) ± 9.7 (standard deviation, s) mg/g of creatinine, whereas nine of ten urine samples from patients with gastric cancer gave high values of N-acetylneuraminic acid that exceed $m + 2s$. The samples from patients with cancer of the lung, liver, pancreas and ovary showed much higher values of N-acetylneuraminic acid, though the number of cases was not sufficient to generalize these observations. There are findings that serum N-acetylneuraminic acid contents are elevated in cancer [9, 10], but reliable data on urinary N-acetylneuraminic acid content have not been published, probably because in the conventional colorimetric methods there is too much interference by accompanying substances. Our experiments indicate that the values obtained by the colorimetric method [3] were several times higher than those obtained by the present method, and in addition the former varied widely with sample size. Since these preliminary data showing the elevation of urinary N-acetylneuraminic acid levels in cancer is suggestive of diagnostic application, further studies are now in progress.

TABLE II

PRELIMINARY DATA ON URINARY N-ACETYLNEURAMINIC ACID LEVELS FOR NORMAL SUBJECTS AND CANCEROUS PATIENTS

No.	Age	Sex	Cancerous organ	Concentration of N-acetyl-neuraminic acid (mg/dl)	Concentration of creatinine (mg/dl)	N-Acetylneura-minic acid/ creatinine ratio (mg/g)
1	41	Female	Stomach	1.79	43.4	41.2
2	52	Male	Stomach	8.78	214	41.0
3	55	Male	Stomach	7.84	187	41.9
4	57	Female	Stomach	4.84	75.7	63.9
5	65	Male	Stomach	1.81	30.6	59.1
6	65	Female	Stomach	3.07	124	24.8
7	68	Female	Stomach	12.9	183	70.5
8	69	Male	Stomach	4.09	96.7	42.3
9	70	Male	Stomach	7.43	118	63.0
10	75	Female	Stomach	3.47	43.1	80.5
11	75	Male	Lung	5.29	70.3	75.2
12	75	Male	Lung	8.70	85.2	102
13	77	Female	Lung	6.03	49.0	123
14	65	Male	Liver	10.4	102	102
15	64	Male	Pancreas	8.27	55.4	149
16	54	Female	Ovary	8.84	56.7	156
Normal, mean ± standard deviation (n = 18)				2.83 ± 1.00	133 ± 54.6	21.2 ± 9.7

REFERENCES

1 A. Gottschalk, Glycoproteins, Their Composition, Structure and Function, Part A, Elsevier, Amsterdam, 1972, p. 403.
2 A.P. Confield, C. Ferreira do Amaral, M. Wember and R. Shauer, Eur. J. Biochem., 68 (1976) 597.
3 L. Warren, J. Biol. Chem., 234 (1959) 1971.
4 L. Svennerholm, Biochim. Biophys. Acta, 24 (1957) 604.
5 D.G. Comb and S. Roseman, Methods Enzymol., 5 (1962) 287.
6 S. Honda, N. Yamauchi and K. Kakehi, J. Chromatogr., 169 (1979) 287.
7 R.W. Bonsnes and H.H. Taussky, J. Biol. Chem., 158 (1945) 581.
8 J. Havercamp, R. Shauer, M. Wember, J.P. Farriaux, J.P. Karmaling, C. Vershuis and J.F.C. Viegenthart, Hoppe-Seyler's Z. Physiol. Chem., 357 (1976) 1699.
9 H.K.B. Silver, D.M. Rangel and D.L. Morton, Cancer, 41 (1976) 1497.
10 T.M. Kloppel, T.W. Keenan, M.J. Freeman and D.J. Morre, Proc. Nat. Acad. Sci. U.S., 74 (1977) 3011.