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Abstract

A new acidic ninhydrin method for determining free sialic acids is described. The method is based on the reaction of sialic acids with Gaitonde's acid ninhydrin reagent 2 which yields a stable color with an absorption maximum at 470 nm. The standard curve is linear in the range of 5 to 500 nmol of N-acetylneuraminic acid per 0.9 ml of reaction mixture. The reaction was specific only for sialic acids among the various sugars and sugar derivatives examined. Some interference of this method by cysteine, cystine and tryptophan was noted, although their absorption maxima differed from that of sialic acids. The interference by these amino acids was eliminated with the use of a small column of cation-exchange resin. The acidic ninhydrin method provides a simple and rapid method for the determination of free sialic acids in biological materials.

KEYWORDS: sialic acid determination, acidic ninhydrin reaction, acidic ninhydrin method

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Determination of Sialic Acids by Acidic Ninhydrin Reaction

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A new acidic ninhydrin method for determining free sialic acids is described. The method is based on the reaction of sialic acids with Gaitonde's acid ninhydrin reagent 2 which yields a stable color with an absorption maximum at 470 nm. The standard curve is linear in the range of 5 to 500 nmol of N-acetylneuraminic acid per 0.9 ml of reaction mixture. The reaction was specific only for sialic acids among the various sugars and sugar derivatives examined. Some interference of this method by cysteine, cystine and tryptophan was noted, although their absorption maxima differed from that of sialic acids. The interference by these amino acids was eliminated with the use of a small column of cation-exchange resin. The acidic ninhydrin method provides a simple and rapid method for the determination of free sialic acids in biological materials.

Key words : sialic acid determination, acidic ninhydrin reaction, acidic ninhydrin method

Many methods for determining sialic acids have been reported (1-8). Among them, methods using periodate-thiobarbituric acid (4), periodate-resorcinol (5), enzymes (neuraminidase (EC 3.2.1.18), N-acetylneuraminic acid-aldolase (EC 4.1.3.3) and pyruvate oxidase (EC 1.2.3.3)) (6) and high-performance liquid chromatography (7) are often applied to biological materials. However, these procedures are intricate and time-consuming.

We report here a simple, specific and rapid method for the determination of sialic acids using an acidic ninhydrin reaction.

Materials and Methods

N-Acetylneuraminic acid, N-glycolylneuraminic acid, N-acetylneuramin-lactose, N-acetylmuramic acid and muramic acid were obtained from Sigma Chemical Co., St Louis, Mo., USA. N-Acetyl-D-glucosamine, D-glucosamine hydrochloride, D-galactosamine hydrochloride, D-glucuronolactone, D-fructose, D-galactose, D-mannose, D-xylose, L-rhamnose and ninhydrin were purchased from Wako Pure Chemical Ind., Ltd., Osaka, Japan. N-Acetyl-D-galactosamine was purchased from Nakarai Chemical Co., Kyoto, Japan.

Male mice of the DBA strain weighing about 25 g were obtained from the Animal Center for Medical Research, Okayama University Medical School.

Acidic ninhydrin reaction with sialic acids. The determination of free sialic acids was performed according to the method of Gaitonde (9) described for the determination of cysteine with some modification as follows. Three tenths ml of glacial acetic acid and 0.3 ml of acid ninhydrin reagent 2 were added to 0.3 ml of a sample solution containing 5 to 500 nmol of sialic acids. The reaction mixture was heated in a boiling water bath for exactly 10 min, and then chilled in an ice-water bath. The absorbance at 470 nm was measured using a micro-cuvette with a light path of 10 mm. The absorbance can also be determined using a usual cuvette after the addition of 3.0 ml of 99% ethanol. The acid ninhydrin reagent 2 of Gaitonde was prepared as described (9).

Periodate-resorcinol reaction with sialic acids.

The periodate-resorcinol reaction with sialic acids was carried out according to the method of Jourdian *et al.* (5).

Isolation of sialic acids from mouse peritoneal fluid. Mice were intraperitoneally inoculated with 1×10^6 Ehrlich ascites tumor cells per animal. The ascites fluid was collected 8 to 10 days after the inoculation. The isolation of sialic acids from

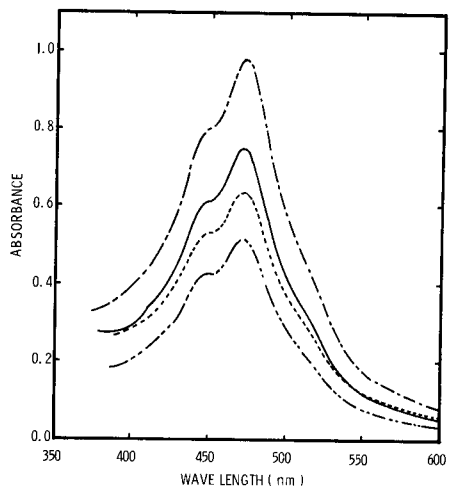

Fig. 1 Absorption spectra of reaction products of sialic acids with acid ninhydrin reagent 2. N-Acetylneuraminic acid, —— ; sialic acid fraction isolated from mouse peritoneal fluid, —·— ; N-acetylneuramin-lactose, —··— ; N-glycolylneuraminic acid, ------ . The concentrations of N-acetylneuraminic acid, N-acetylneuramin-lactose and N-glycolylneuraminic acid were 100, 100 and 77.6 nmol per incubation mixture, respectively.

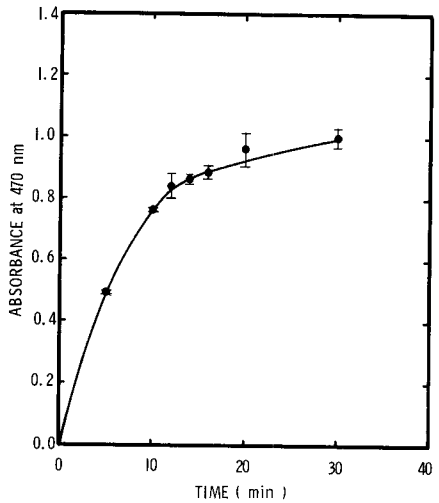

Fig. 2 Effect of the heating time on the color yield of the acidic ninhydrin reaction with N-acetylneuraminic acid. The reaction was performed according to the standard procedure described under Materials and Methods. Each value is the mean ± SD of three determinations.

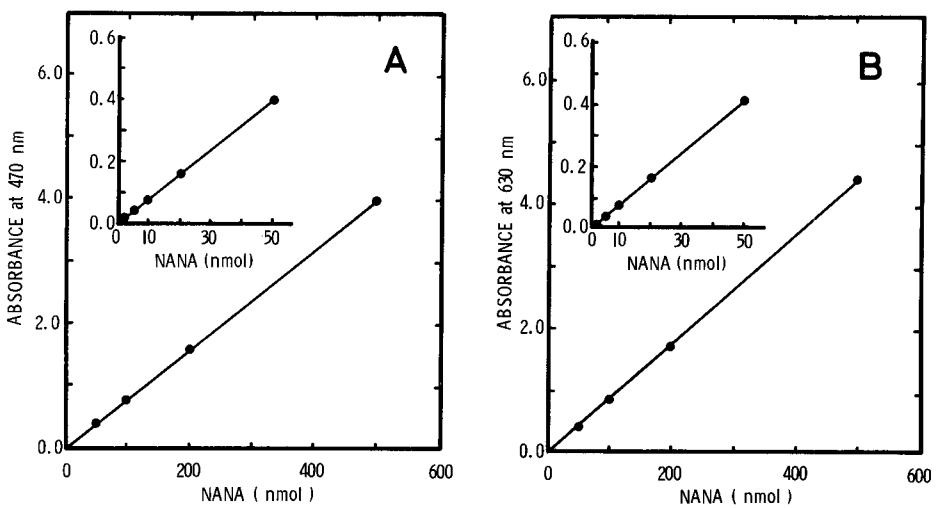

Fig. 3 Standard curves of N-acetylneuraminic acid (NANA) determined by the acidic ninhydrin method (A) and by the periodate-resorcinol method (B).

the acid soluble fraction of the peritoneal fluid was performed according to the method of Carubelli *et al.* (10).

Results

Figure 1 shows the absorption spectra of the products of the acidic ninhydrin reaction with authentic N-acetylneuraminic acid, N-glycolylneuraminic acid, N-acetylneuraminlactose and a sialic acid fraction isolated from the mouse peritoneal fluid. These materials all exhibited the same absorption profile and an absorption maximum at 470 nm.

The effect of the heating time on the acidic ninhydrin reaction with N-acetylneuraminic acid is illustrated in Fig. 2. The absorption at 470 nm increased proportionally with the incubation time up to 10 min and increased gradually thereafter until reaching a maximum at about 30 min. The color yield at 10 min was approximately 75% of that at 30 min. Up to 10 min, the absorbance at a definite incubation time was constant among determinations performed at the same time. However, the color yields at the longer reaction times tended to vary as shown in Fig. 2. Therefore, a 10 min heating time was employed in the standard assay procedure. The color yielded by heating for 10 min was quite stable. The decrease in the absorbance was less than 4% after standing at 0°, 16° or 37℃ for 3 h. Fig. 3A shows the standard curve of N-acetylneuraminic acid determined by the present method. The curve was linear up to 500 nmol of N-acetylneuraminic acid. Fig. 3B is the standard curve of N-acetylneuraminic acid determined by the periodate-resorcinol method (5). The specificity of the acidic ninhydrin reaction was examined using various compounds listed in Table 1. Only N-acetylneuraminic acid and neuraminic acid-containing compounds (see also Fig. 1) produced the color exhibiting an absorption maximum at 470 nm. The molar extinction coefficient of N-acetylneuraminic acid under the present conditions was 6.64×10^3. Table 1 also shows the effect of various compounds on the acidic ninhydrin reaction with N-acetylneuraminic acid. L-Cysteine, L-cystine, L-proline and L-tryptophan reacted with acid ninhydrin reagent 2, and yielded colors showing absorption maxima at 560 (9), 485 (9), 510 (9) and 385 nm, respectively. These amino acids affected the absorbance at 470 nm as shown in the table, and their color yields at 470 nm were additive. The interference by these amino acids could be elimi-

Table 1 Specificity of the acidic ninhydrin reaction and effect of various compounds on the reaction with N-acetylneuraminic acid[a]

Substances	Absorbance at 470 nm	
		+N-acetylneuraminic acid
N-acetylneuraminic acid	0.369	-
N-Acetyl-D-glucosamine	0.003	0.374
N-Acetyl-D-galactosamine	0.005	0.374
N-Acetylmuramic acid	0.003	0.373
Muramic acid	0.003	0.378
D-Glucuronic acid	0.003	0.378
D-Glucosamine	0.003	0.375
D-Galactosamine	0.002	0.380
D-Fructose	0.002	0.360
D-Galactose	0.002	0.385
D-Mannose	0.001	0.391
D-Mannitol	0.002	0.370
D-Xylose	0.002	0.369
L-Rhamnose	0.004	0.370
2-Deoxy-D-ribose	0.006	0.367
L-Cysteine	0.266	0.596
L-Cystine	0.083	0.423
L-Tryptophan	0.030	0.413
L-Histidine	0.006	0.380
L-Proline	0.011	0.371
Glutathione (reduced)	0.006	0.364
Urocanic acid	0.001	0.365
Anthranilic acid	0.002	0.377
Niacine	0.002	0.372
Uric acid	0.002	0.371
Uracil	0.002	0.369

a: N-Acetylneuraminic acid and the other substances were used at a concentration of 50 nmol per 0.9 ml of reaction mixture in the standard procedure described under Materials and Methods.

Table 2 Determinations of sialic acids in a sialic acid fraction from mouse ascites fluid by the acidic ninhydrin method and by the periodate-resorcinol method[a]

Method	Sialic acid (nmol/ml)
Acidic ninhydrin method	158.7 ± 2.7
Periodate-resorcinol method	159.1 ± 4.4

a: The acidic ninhydrin method was performed as described under Materials and Methods. The periodate-resorcinol method was performed according to the method of Jourdian *et al.* (5). Each value is the mean ± SD of ten determinations.

nated with the use of a cation-exchanger column. A mixture of 200 nmol each of N-acetylneuraminic acid, cysteine and tryptophan was applied to a column of Dowex 50 (H^+ form, 0.7×2.5 cm), which was washed 4 times successively with 0.5 ml-portions of water. The initial effluent and washings were combined and subjected to the acidic ninhydrin reaction. The recovery of N-acetylneuraminic acid by this procedure was 101.5 ± 2.0%, and amino acids were not detected in the effluent. When the sialic acid content was too low to make an accurate determination, the effluent was concentrated using a centrifugal concentrator. The present method was applied to the sialic acid determination of a sialic acid fraction obtained from mouse ascites fluid. The values were compared with those obtained by the periodate-resorcinol method. These two values agreed well as shown in Table 2.

Discussion

The acidic ninhydrin reaction was developed by Chinard for the determination of proline and ornithine (11). Gaitonde modified the reaction and prepared acid ninhydrin reagent 2, which was applied to the determination of cysteine in biological materials in the presence of other naturally occuring amino acids (9). In the present study, it was found that acid ninhydrin reagent 2 reacted with sialic acids, and the method could be applied to the determination of sialic acids. The reaction was specific only for N-acetylneuraminic acid and other neuraminic acid-containing compounds among the sugars tested. The reaction was not interfered with by any of the sugars which do not contain neuraminic acid as shown in Table 1. Amino acids such as cysteine, cystine, proline and tryptophan react with acid ninhydrin reagent 2. However, the absorption maxima of these compounds are different from that of sialic acids. Nevertheless, when cysteine, cystine and tryptophan are contained in samples in high concentrations, they may interfere with the accurate determination of sialic acids. These amino acids can be eliminated by passing the sample solution through a small column of Dowex 50 (H^+ form).

The sensitivity of the reaction was quite high. In the standard procedure, as little as 5 nmol of N-acetylneuraminic acid in 0.3 ml of sample solution was determined accurately. Thus, the sensitivity is comparable with that of the periodate-resorcinol reaction which has been used widely. However, the present method is much simpler than the periodate-resorcinol method, and the time required for the whole process is less than half of that required for the latter method. Moreover, the color developed is stable. Therefore, determinations of sialic acids in many samples can be performed easily at the same time.

As shown in Fig. 1, the absorption spectrum produced by the present reaction with a sialic acid fraction obtained from mouse ascites fluid was the same as that with authentic sialic acids. The values of sialic acids determined by the present method and by the periodate-resorcinol method agreed well as shown in Table 2. These results indicate that the acidic ninhydrin method is specific and accurate. Thus, the present method may be useful for the determination

of free sialic acids in biological materials.

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