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Short Communication

Determination of N-acetylneuraminic acid and N-glycolylneuraminic acid in glycoproteins by high-performance liquid chromatography without derivatization

Haruko K. Ogawa, Yoshie Takeuchi, Haruhi Uchibori, Isamu Matsumoto and Nobuko Seno

Department of Chemistry, Faculty of Science, Ochanomizu University, Bunkyo-ku, Tokyo 112 (Japan)

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ABSTRACT

A simple and sensitive method for the determination of N-acetylneuraminic acid and N-glycolylneuraminic acid in glycoproteins by high-performance liquid chromatography has been investigated. The best conditions to release sialic acid from glycoproteins involved incubation at 80°C for 1 h *in vacuo* in 0.01 *M* hydrochloric acid with removal of the hydrochloric acid by evaporation without decomposing the sialic acids. Of the substances examined, N-acetylglycine was the best internal standard, because it is absent from glycoproteins, has an appropriate retention time on high-performance liquid chromatography (HPLC) and is stable during the procedures. The sample solution containing the internal standard was analysed directly by isocratic HPLC without pre-column or post-column labelling, and elution was monitored by ultraviolet absorbance at 205 nm. This method can determine 0.08–10 nmol of sialic acid accurately within 12 min and was successfully applied to bovine vitronectin.

INTRODUCTION

Sialic acids in glycoproteins are important not only as clinical markers of inflammation and tumours, but also as biological signals for clearance, cellular recognition and adhesion [1,2].

The two most predominant sialic acids in nature, N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA), have been separated and identified by thin-layer chromatography, gas chromatography and high-performance liquid chromatography (HPLC). Of these methods, HPLC is useful for the identification and determination of individual sialic acids because of its sensitivity and high resolution [3–10]. Pre-column or post-column labelling methods provide higher specificity and sensitivity but require derivatization steps or complicated HPLC systems that usually require two pumps and a reaction bath. The simplest system comprises direct photometric detection in the near-UV region, which has the advantage of picomolar sensitivity [6,10]

Correspondence to: Haruko K. Ogawa, Department of Chemistry, Faculty of Science, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112, Japan.

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but is subject to interference by accompanying substances. When large amounts of solvents are required for hydrolysis of minute amounts of sample, *e.g.* "on-membrane" analysis from Western-blotted membranes, the tailing of the solvent acid peak interferes with the accurate estimation of sialic acid peaks [11]. This problem, however, can be circumvented by choosing hydrolysis conditions selective for sialic acid.

In order to establish the simplest system for the rapid and direct determination of sialic acids in glycoproteins, we first searched for an appropriate internal standard for this HPLC system, as D-ribose is for most analyses of neutral sugars. Next, the utility of volatile acids for hydrolysis, which would allow solvent removal and concentration of the hydrolysate by evaporation, was investigated. The applicability of this improved method is shown for a glycoprotein, vitronectin, from bovine plasma, which contains both NANA and NGNA in the same molecule [11,12].

EXPERIMENTAL

Chemicals

Bovine vitronectin was purified from bovine plasma according to Yatohgo *et al.* [13]. NANA and NGNA were obtained from Nakarai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, USA), respectively. Sulphuric acid, hydrochloric acid, acetic acid and trifluoroacetic acid were superspecial grade and amino acid sequence grade from Wako (Osaka, Japan). All other chemicals used were special grade unless otherwise specified.

Hydrolysis of glycoprotein samples

Bovine vitronectin (30 μg) was dissolved in 50 μl of 0.05 *M* sulphuric acid or 0.1 or 0.01 *M* hydrochloric acid in a tapered glass tube (0.8 × 9 cm). The glass tube was evacuated for 30 s, then sealed and heated for 1 h at 80°C. The reaction solution was cooled immediately to room temperature and centrifuged at 1000 *g*, and the tube was opened. After the internal standard had been added, the solution was mixed well and centrifuged, and the supernatant was injected directly onto the HPLC column. For evaporation, the solution was transferred to another tube and concentrated *in vacuo* at 36–40°C, and the residue was dissolved in a suitable volume of water. In each case, the sample solution was filtered through Ultrafree C3GV (pore size 0.22 μm, Millipore) before injection.

High-performance liquid chromatography

The HPLC system consisted of a Tosoh HLC 803D pump, a Rheodyne injector with a 20-μl loop, a column oven and a Tosoh UV8000 spectrophotometer with a 20-μl cell. Separation of sialic acids by ion-exclusion chromatography was performed using a cation-exchange column of Shodex Ionpak KC-811 (300 mm × 8 mm I.D., Showa Denko, Tokyo, Japan) with a guard column of Shodex KC-810. Elution was carried out at 60°C with 0.3% phosphoric acid at a flow-rate of 1.0 ml/min; the sialic acids were monitored at 205 nm.

Selection of internal standards

N-Acetylglycine, glycine and D-gluconic acid (10 μg/ml) were injected separately on to the HPLC column to obtain their elution positions. The separation profile was monitored by injecting a mixture of the three compounds (3.5 nmol of each) with NANA and NGNA in 0.05 *M* sulphuric acid (1.5 nmol of each sialic acid per 10 μl).

Acid hydrolysis

Acids generally used to liberate sialic acid [1,14] were compared for their utility in achieving high sensitivity in this HPLC system. The elution positions of acid were tested by injecting 10 μl each of 0.05 *M* sulphuric acid, 0.1 *M* hydrochloric acid, 0.01 *M* hydrochloric acid, 2 *M* acetic acid or 10% trifluoroacetic acid separately. Then NANA and NGNA were dissolved in each acid (7.5 nmol of each sialic acid per 40 μl), and N-acetylglycine (17.5 nmol per 10 μl) was added as an internal standard after incubation at 80°C for 60 min in an evacuated sealed tube. Aliquots of 10 μl of each mixture were injected.

Effect of evaporation

The standard mixtures of NANA and NGNA (each 0.25–1.5 nmol per 10 μl) with N-acetylglycine (3.5 nmol per 10 μl) in 0.1 or 0.01 *M* hydrochloric acid were dried *in vacuo* at 36–40°C within 20 min and dissolved in 20 μl of water. Aliquots of 10 μl of each solution before and after drying were injected onto the HPLC column.

RESULTS

High-performance liquid chromatography

A baseline separation of standard NANA and NGNA could be achieved within 8 min under the conditions of this HPLC system. Higher concentrations of phosphoric acid, up to 0.5%, and higher temperatures, up to 60°C, gave a slightly better separation of sialic acids. At 60–75°C, the separation did not change by more than 0.05 min in the range 0.3–1% phosphoric acid. Because of the acid lability of sialic acids, 0.3% phosphoric acid was used for elution at 60°C in all the experiments.

Selection of internal standards

As shown in Fig. 1, the acid (sulphuric acid or hydrochloric acid), NGNA and NANA eluted at 5, 6 and 7 min, respectively, while N-acetylglycine eluted at 12 min. Glycine and gluconic acid (not shown) eluted at 5 and 7 min, respectively. Only N-acetylglycine, therefore, was well separated from the sialic acid peaks. The calibration curve (mol *versus* peak area) for N-acetylglycine was linear over the range of 1–5 nmol.

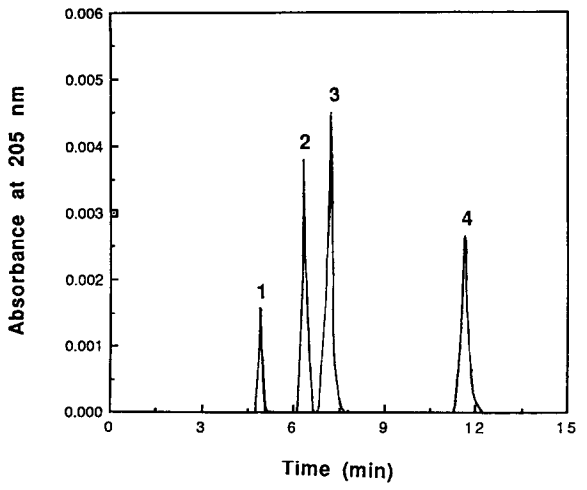

Fig. 1. Chromatogram of a mixture of NANA, NGNA and N-acetylglycine in 0.01 *M* hydrochloric acid. A portion (10 μl) of a mixture contained 1.5 nmol each of NANA and NGNA and 3.5 nmol of N-acetylglycine. Peaks: 1 = acid injection peak; 2 = NGNA; 3 = NANA; 4 = N-acetylglycine.

Stability of sialic acids and N-acetylglycine under hydrolysing conditions

For sialic acid analysis, hydrolysis is usually carried out at 80°C in 0.05 *M* sulphuric acid for 60 min or 0.1 *M* sulphuric acid for 30 min [1,3]. However, for dilute glycoprotein samples or direct analysis of glycoproteins electroblotted onto suitable membranes, the use of a volatile acid is necessary. Of the volatile acids tested, 2 *M* acetic acid and 10% trifluoroacetic acid eluted at the same position as N-acetylglycine. When 0.01 *M* hydrochloric acid was used, the peaks were separated as well as with 0.05 *M* sulphuric acid, as shown in Fig. 1. The calibration curves (molar ratio *versus* area ratio) for NANA and NGNA were linear over the range 0.08–10 nmol for both acids with a slope of 3.3 and 1.8, respectively. Therefore, hydrochloric acid was used as the volatile acid for hydrolysis and tested for evaporation.

If 0.1 *M* hydrochloric acid was used, the acid peak was large compared with the same volume of 0.05 *M* sulphuric acid or 0.01 *M* hydrochloric acid, and overlapped with the sialic acid peaks. To reduce the tailing of the acid peak and to concentrate the hydrolysate, the hydrochloric acid was removed by evaporation. The peak of the acid was markedly reduced in size by a single evaporation in the case of both 0.1 and 0.01 *M* hydrochloric acid. The calibration curves for NANA and NGNA in hydrochloric acid before and after evaporation were reproducible within ± 5% in triplicate experiments. The difference between the curves in 0.01 *M* hydrochloric acid before and after evaporation was negligible and the curves were similar to those of the 0.05 *M* sulphuric acid hydrolysate, suggesting that the internal standard was stable under these conditions. In contrast, the curves for each sialic acid

hydrolysed in 0.1 *M* hydrochloric acid differed greatly before and after evaporation, with a 1.3-fold increase in each slope after evaporation, a result thought to arise from estimation errors caused by the tailing of the acid peak and the increased lability of N-acetylglycine during evaporation in 0.1 *M* hydrochloric acid. On the basis of these results, 0.01 *M* hydrochloric acid was used for hydrolysis, followed by evaporation to improve sensitivity.

Applications

Purified bovine vitronectin was hydrolysed in 0.01 *M* hydrochloric acid and analysed as described, using N-acetylglycine as an internal standard as shown in Fig. 2. The amounts of NGNA and NANA released from 1 mol of bovine vitronectin (based on a calculated molecular weight of 60 kDa [15]) were 9.0 and 4.7 mol, respectively, both before and after evaporation. These values are in good agreement with the values of 9.5 and 5.3 mol, respectively, obtained by hydrolysis with 0.05 *M* sulphuric acid and direct injection onto the HPLC column. The molar ratios of NGNA to NANA were thus 1.9 by 0.01 *M* hydrochloric acid hydrolysis and 1.8 by 0.05 *M* sulphuric acid hydrolysis. Bovine vitronectins purified from other bovine sera had different sialic acid compositions, with molar ratios of NGNA to NANA varying from 1.9 to 0.7; these ratios were reproducible, which suggests the heterogeneity of sialylation among individual sera within one species.

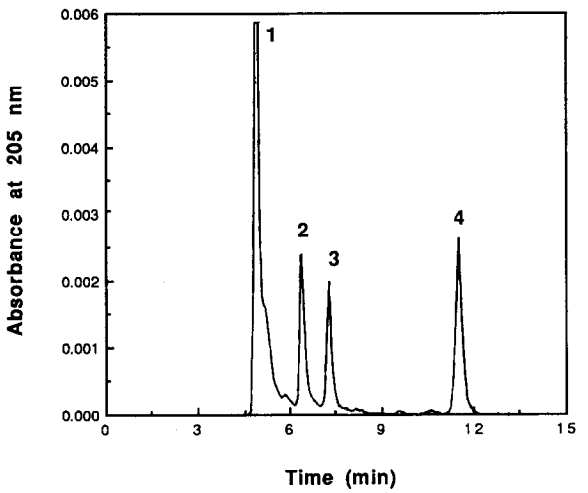

Fig. 2. Chromatogram of the hydrolysate of bovine vitronectin. Bovine vitronectin (30 μg) was hydrolysed in 40 μl of 0.01 *M* hydrochloric acid for 1 h at 80°C. After N-acetylglycine (17.5 nmol per 10 μl) was added, the supernatant (10 μl) was injected directly onto the HPLC column. The elution was performed as described in the text. Peaks: 1 = acid injection peak; 2 = NGNA; 3 = NANA; 4 = N-acetylglycine.

DISCUSSION

By directly injecting the hydrolysate and using N-acetylglycine as an internal standard, separation and determination of subnanomolar levels of NANA and NGNA was achieved within 12 min using a simple HPLC system without special apparatus such as a pulsed amperometric detector. In this study, the internal standard and acid for hydrolysis were varied to find the optimal conditions under which both NANA and NGNA could be assayed in a single chromatographic step, in order to widen the applicability of the method to glycoproteins from other animal sources.

Silver *et al.* [6] used N-acetylglucosamine as an internal standard and 0.05 *M* sulphuric acid for hydrolysis to analyse NANA in human serum by ion-exclusion HPLC with UV detection. We have used N-acetylglycine as an internal standard because it is better separated from NANA than is N-acetylglucosamine (9 min) under the HPLC conditions described here, and because it does not occur naturally in glycoproteins. The use of 0.01 *M* hydrochloric acid for hydrolysis was suitable when evaporation was performed after hydrolysis to reduce the size of the acid peak and concentrate the sample. Hara *et al.* [16] reported the HPLC of sialic acids with fluorimetric detection, after derivatization of the hydrolysate with 1,2-diamino-4,5-methylenedioxybenzene, to have a high sensitivity of 23 fmol. Their method is the most sensitive of those hitherto reported; our method, however, is more rapid and simpler, does not require derivatization of the sialic acids and has an appropriate internal standard.

With UV detection, consideration must be given to possible interference by other compounds, a

factor that will depend largely on the hydrolysis conditions. Neutral and amino sugars would elute at 8 min in this HPLC system. However, these compounds are hardly liberated under the hydrolysis conditions used in this study [14], and they have very low UV absorbances (1:1000) compared with sialic acids [4]. Amino acids, especially aspartic acid, are released by hydrochloric acid concentrations below 0.1 *M* at 105°C [17], but there are no data regarding release at 80°C. Aspartic acid at levels up to 1 μmol produced no peak under the present detection conditions. There still remains the possibility that some other acid-labile peptide bond might be cleaved under these hydrolysis conditions. For bovine serum albumin, however, no interfering peaks were observed in HPLC after hydrolysis (data not shown). Among intact glycoproteins, vitronectin and transferrin eluted at the same position as the acid peak and fetuin eluted faster than the acid peak at 3.0–3.7 min. Thus, we could not find interfering substances among glycoprotein hydrolysates.

Although we did not demonstrate the specificity of UV detection for sialic acid, Silver *et al.* [6] also detected no interfering substances in human serum hydrolysates. Furthermore, the sialic acid content of human vitronectin measured by this method, when combined with the hexosamine and neutral carbohydrate contents, agreed well with the structure of its major oligosaccharide ($NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$) [18]. As previously reported [11], PVDF membranes produced no interfering peaks under these HPLC conditions; thus, this improved method can also be applied to "on-membrane analysis".

These results indicate that this HPLC method is reliable; its simplicity will make it valuable for both biochemical and clinical use.

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