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Simple fluorimetric method for quantification of sialic acids in glycoproteins

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Abstract

A simple and rapid fluorimetric method was developed for detection and quantitative analysis of sialic acids in glycoproteins. Sialic acid residues in glycoproteins were specifically oxidized with periodate at 0 °C for 45 min. Formaldehyde generated from carbon 9 (C-9) of sialic acid was converted specifically to fluorescent dihydropyridine derivative with acetoacetanilide and ammonia at room temperature for 10 min. The reaction products indicate intense fluorescence with excitation and emission maxima at 388 and 471 nm, respectively. When the reaction was conducted in approximately a 1-ml volume, the linearity of the calibration exhibited between 2 and 180 μg of bovine fetuin, or between 0.3 and 27 nmol of *N*-acetylneuraminic acid, as a model glycoprotein. The limit of detection, based on three times the standard deviation of the reagent blank, was 0.5 μg of fetuin. The proposed method was applied to determination of sialic acids in various glycoprotein samples. This proposed method is simple and obviates the heating and extraction steps. It is highly specific to sialic acids in glycoproteins and indicates no fluorescence of neutral glycoproteins.

Keywords: Hantzsch reaction; Periodate oxidation; Sialic acid; Neuraminic acid; Fluorimetry; Glycoprotein

The term sialic acid indicates a general name for nine carbon acidic sugars that includes *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, and 2-keto-3-deoxy-D-glycero-D-galactonononic acid (α-D-KDN)[1] [1,2]. Sialic acid is widely distributed throughout human tissues and is present in several fluids, including serum, cerebrospinal fluid, saliva, urine, amniotic fluid, and breast milk, and it is found in high levels in the brain, adrenal glands, and heart [3–7]. Most neuraminic acids are present at the outermost ends of N- and O-linked oligosaccharides in glycoproteins and glycosphingolipids. They play an important role in expressing the functions of these glycoconjugates [8–12]. Sialic acid enhances the viscosity of mucus to prevent bacterial and viral infection. In several in vitro and animal studies, the sialylated oligosaccharides have been shown to inhibit strains of influenza A and B viruses more effectively than any prescription antiviral agent, and they also influence blood coagulation and cholesterol levels [13]. Sialic acid metabolism abnormalities induce severe diseases [14,15]. Moreover, sialic acid contents in body fluids become a means to diagnose cancer in patients because alteration of glycans reflects the influence of various types of tumors [16]. Consequently, a reliable simple method to determine sialic acid is needed.

Many procedures have been developed for detection and quantification of sialic acids in glycoconjugates [17]. A widely used colorimetric assay that is both specific and sensitive for free sialic acid is based on periodate oxidation followed by reaction with 2-thiobarbituric acid. This procedure, which was developed independently by Warren [18] and Aminoff [19], uses a reaction based on the eventual formation of β-formyl pyruvic acid from free sialic acid by periodate oxidation. The reaction includes 2 mol of thiobarbituric acid with 1 mol of β-formyl pyruvic acid to yield a chromophore with absorption maximum at 549 nm

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[1] *Abbreviations used:* α-D-KDN, 2-keto-3-deoxy-D-glycero-D-galactononic acid; C-9, carbon 9.

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[20,21]. This method is sensitive, but sialic acid must be released from glycoconjugates before analysis. The resorcinol periodate method is another version used for sialic acid determination that enables the quantitation of sialic acid that is bound covalently to glycoconjugates [22]. However, this method is not sensitive. Insensitivity of the spectrophotometric methods is subject to interference from coexisting substances. Anion exchange HPLC with pulsed amperometric detection is used frequently for determination of free *N*-acetyl and *N*-glycolylneuraminic acids and their oligosaccharides [3], but sometimes it is subject to interference by contaminants in the sample. Postcolumn HPLC using cyanoacetamide was developed for determination of *N*-acylmannosamines derived enzymatically from neuraminic acids [23]. Subsequent HPLC with fluorimetric detection after labeling with 1,2-diamino-4,5-methylenedioxybenzene enables sensitive and specific detection of free sialic acid with a detection limit of 25 fmol [24]. This method was further applied to simultaneous determination of approximately 13 members of the sialic acid family with substitutions such as *N*-acetyl, *O*-acetyl, *N*-glycolyl, and sulfate, and it was applied for analyses of sialic acids in tissues of mice and rats [25]. In addition, HPLC methods can provide information related to the chemical structure of sialic acids in glycoconjugates, but such methods require a long analysis time as well as expensive equipment and columns.

Recently, Li and coworkers reported acetoacetanilide for sensitive detection of formaldehyde in environmental samples [26]. The reaction proceeds at room temperature. The product can be detected fluorimetrically using the lower level of detection of 10^{-8} M. For the current study, we applied this reaction by introducing a periodate oxidation step before the reaction with acetoacetanilide reagent. The reaction was based on the Hantzsch reaction, which includes simultaneous condensation of one molecule of ammonia, two molecules of acetoacetanilide, and one molecule of formaldehyde generated from carbon-9 (C-9) of sialyl residues by periodate oxidation (Scheme 1). This method can determine sialic acids bound to glycoconjugates without acid hydrolysis. The method is simple; the reaction was carried out in an ice bath and at room temperature. All procedures can be completed within 1 h.

Materials and methods

Instrumentation

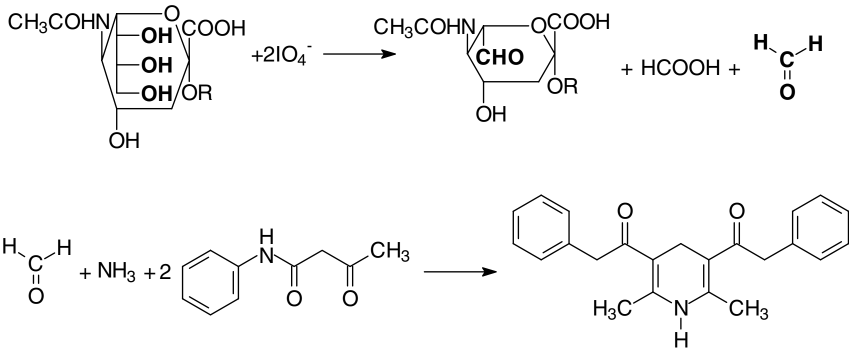
All fluorimetric measurements were carried out using a spectrofluorometer (FP-6200, Jasco) equipped with a xenon lamp, dual monochrometers, and a controlling computer with operating software (Microsoft Windows). The slit widths for both excitation and emission were set at 5 nm. The sample solution was transferred to a conventional 1 × 1-cm quartz cell and then mounted on a cell holder. Subsequently, fluorescence spectra and their associated intensities were observed using the standard method. All measurements were performed in a temperature-controlled room (25 ± 1 °C). Reaction mixtures obtained using the thiobarbituric acid method were measured using a spectrophotometer (V630, Jasco).

Materials

Acetoacetanilide, ammonium acetate, an aqueous solution of formaldehyde (37%), sodium periodate, and sodium thiosulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine pancreas ribonuclease B, hen ovalbumin, human fibrinogen, porcine thyroglobulin, porcine stomach mucin, human transferrin, human α_1-acid glycoprotein, and *N*-acetylneuraminyl lactose (NeuAcα2-3Galβ1-4Glc) were obtained from Sigma–Aldrich (Tokyo, Japan). *N*-Acetylneuraminic acid and other carbohydrate specimens were obtained from Nacalai Tesque (Kyoto, Japan). Fetal calf serum fetuin was obtained from Gibco Oriental (Tokyo, Japan). Other chemicals and solvents were of the highest commercially available grade.

Standard procedure for determination of sialic acid

All solutions were precooled in an ice bath. Sodium periodate solution (10 mM, 20 µl) was added to 200 µl of glycoconjugate sample (10–200 µg) placed in a 10-ml polypropylene test tube. The solution was chilled in the ice bath for 45 min. Adding 100 µl of 50 mM sodium thiosulfate solution terminated the reaction. To the reaction mixture were added 500 µl of 4.0 M ammonium acetate



Scheme 1. Periodate oxidation of sialic acid and reaction of formaldehyde with acetoacetanilide.