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Determination of sialic acids by liquid chromatography–mass spectrometry

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Abstract

Sialic acids are widely found in nature as components of oligosaccharide units in mucins, glycoproteins and other microbial polymers. Existing methods for determining these acids are long, tedious, and not specific. A simple, rapid, and sensitive method for determining the most commonly occurring acids, *N*-acetylneuraminic and *N*-glycolylneuraminic acid, using LC–MS is described. Standard solutions of the sialic acids with the internal standard, *N*-acetylneuraminic acid methyl ester, were quantitatively analyzed by positive ion electrospray ionization. Fetuin was used as a model glycoprotein and the hydrolysate was injected directly onto an ES Industries AquaSep 3 μm 150×4.6 mm column eluted with a 0.1% aqueous formic acid mobile phase at a flow-rate of 0.5 ml/min. Detection was achieved using the Finnigan Navigator MS system in the selected ion monitoring mode for the protonated molecular ions at *m*/*z* 310, 324, and 326. The linearity over the dynamic range 10 to 1000 ng of sialic acids on-column had a correlation coefficient greater than 0.999. The amount of sialic acids found in the fetuin hydrolysate was in agreement with values reported in the literature.

Keywords: Sialic acids; Fetuin; Glycoproteins; Proteins; Neuraminic acids

1. Introduction

The sialic acids are a group of compounds consisting of N- and O-substituted derivatives of neuraminic acid, a nine carbon polyhydroxaminoketoacid sugar (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosic acid). Sialic acids are distributed widely in nature as the components of the oligosaccharide units in glycolipids, glycoproteins, gangliosides, and mucins [1–4]. Sialic acids in glycoproteins are important as clinical markers of inflammation and tumors. Increased serum levels of sialic acids have been observed in cancer patients [5–10] and patients with congenital metabolic disorders [11,12]. The sialic acids are biological signals for cellular recognition, adhesion, and clearance, e.g. follicle-stimulating hormone, thyroid-stimulating hormone, and C.hivn. 1-NH (esterase inhibitor) [8]. The two most predominant sialic acids occurring in nature are *N*-acetylneuraminic acid (NANA) and *N*-glycolylneuraminic acid (NGNA) (Fig. 1). A number of compounds have been identified for the O-substituted derivatives at the 4, 7, 8, and 9 positions and they will not be discussed in this paper.

Sialic acids can be released from glycoconjugates, which is necessary for their separation and determination, by employing acid hydrolysis or enzymatic techniques. If hydrolysis is used, it is important to minimize decomposition due to hydrolysis itself. The best conditions to release sialic acid from glycopro-

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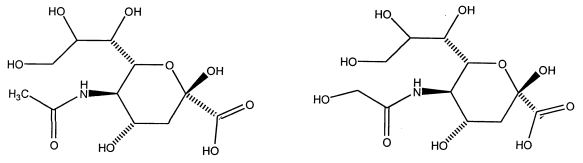

Fig. 1. Structures of the two most predominant sialic acids: *N*-acetylneuraminic acid (NANA; M_r 309.3) and *N*-glycolyneuraminic acid (NGNA; M_r 325.3).

teins involves incubation at 80°C for 1 h in vacuo in 0.01 *M* HCl with removal of HCl by evaporation without decomposition [13]. The neuraminic acids can also be released in the presence of 2 *M* acetic acid by means of microwave hydrolysis, where only 10 min is required [14]. The enzymatic methods using neuramidase takes 18 h at 38°C in 0.05 *M* sodium acetate (pH 5.0) [15]. After hydrolysis, sensitive methods for the quantitative determination of monosaccharides, sialic acids, and amino sugar alcohols can be employed, but they frequently require the use of derivatization techniques followed by high-performance liquid chromatography with fluorescence detection. Fluorescent quinoxalinone derivatives of sialic acid can be separated on a C_{18} reversed-phase column and monosaccharides can be derivatized with anthranilic acid and separated using the same conditions [16]. In contrast to high-performance anion-exchange chromatography with pulsed amperometric detection [17], the fluorescent method is easy to set up for an analysis and offers higher sensitivity than amperometry. Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) was used to define over 30 sialic acids [18]. This method used ESI-MS to monitor the HPLC separation of the 1,2-diamino-4,5-methylenedioxybenzene derivatives. This approach requires no previous purification but involves derivatization reactions followed by direct injection on a microbore HPLC column. A complete spectrum, including molecular ions and column induced dissociation (CID) fragments of sialic acid quinoxalinone, was obtained. Older, more tedious, methods are the periodate–resorcinol colorimetric method, thin-layer chromatography, and gas chromatography [19–21].

The present study is based on the use of a reversed-phase HPLC column designed for use with a highly aqueous mobile phase which enables highly water-soluble solutes to fully interact with bonded C_8 groups. These columns can retain the very hydrophilic sialic acids. Higher specificity and sensitivity for the identification and determination of the individual sialic acids is achieved by employing mass spectrometry in the selected ion monitoring mode. Pre-column or post-column derivatization methods are not necessary.

2. Experimental

2.1. Materials and chemicals

An ES Industries AquaSep 3 μm, 150×4.6 mm column (ES Industries, West Berlin, NJ, USA) was used. The mobile phase was delivered by a Thermo Separations Model P4000 pump and samples were injected by the Thermo Separations autosampler Model P4000 (Thermo Separations Products, San Jose, CA, USA). Detection was performed by a Finnigan Navigator mass spectrometer using ESI for ion production. Formic acid and hydrochloric acid (both GR grade) were obtained from EM Science (Gibbstown, NJ, USA). *N*-Acetylneuraminic acid (sialic acid; NANA) Type VI from *Escherichia coli*, minimum 98%, *N*-glycolylneuraminic acid (NGNA), approximately 90%, *N*-acetylneuraminic methyl ester, minimum 98%, and fetuin from fetal calf serum (F3004) were obtained from Sigma (St. Louis, MO, USA). Water was purified by the Milli-Q UF Plus system by Millipore (Bedford, MA, USA).

Kimax screw cap culture tubes, 13×100 mm, with PTFE-faced rubber lined caps (VWR, Bridgeport, NJ, USA) were used for hydrolysis. The tubes were heated using the Pierce Reacti-Therm heating module (Pierce, Rockford, IL, USA).

2.2. Hydrolysis procedure

Approximately 10 mg of fetuin was transferred to each of five culture tubes. Four milliliters of 0.01 *M* HCl was added to each tube and then the tubes were flushed with a stream of nitrogen for 1 min. The tubes were capped, sealed with PTFE tape and placed into the Reacti-Therm heating block containing a thermometer graduated in 1°C intervals. The heating block was maintained at 80±1°C. After

60 min, the tubes were allowed to cool and the hydrolysate was quantitatively transferred to a 10 ml volumetric flask that contained 1 ml of the internal standard (I.S.), *N*-acetylneuraminic acid methyl ester, concentration 100 μg/ml. The solutions were quantitatively diluted with Milli-Q water.

2.3. Liquid chromatography procedure

The mobile phase was degassed with helium before use. Chromatography was carried out at 40°C at a flow-rate of 0.5 ml/min with 0.1% (v/v) aqueous formic acid as the mobile phase. Ten microliters was injected. The column outlet was connected directly to the mass spectrometer.

2.4. Mass spectrometry procedure

Electrospray ionization was performed in the positive mode with a nitrogen gas flow of 440 l/h. For tuning, the selected ion monitored at *m/z* 310, corresponding to NANA, was optimized by injecting a constant flow of NANA at 100 μl/min into the mobile phase eluting from the column. The response was optimum with a 3.50 kV capillary voltage, a source voltage of 19 V, and source temperature of 180°C. The instrument was operated at unit resolution in the selected ion monitoring (SIM) mode, scanning the masses 310 (NANA), 324 (I.S.), and 326 (NGNA) with a dwell time of 0.300 and mass span of 1.0. The Navigator LC–MS system was interfaced with a computer workstation running ThermoQuest's Excaliber 1.1 software.

2.5. Quantitative determination

Separate calibration curves for NANA and NGNA were established using the internal standard technique. The peak areas from the reconstruction ion chromatograms, after Gaussian smoothing of seven points, for *m/z* 310, 324, and 326 were calculated for calibration levels ranging from 5 to 100 μg/ml, which is equivalent to 50 to 1000 ng injected on column. Each calibration level was analyzed five times. The average peak area response ratios were used to obtain the calibration curve for NANA and NGNA. The slope from the linear regression of the calibration curve was used to calculate the amounts of sialic acid in the fetuin hydrolysate. The average, $n = 5$, peak area response ratio for each of the five fetuin hydrolysate samples was determined. In addition, the recovery was determined for five levels of sialic acids that were subjected to the hydrolysis conditions.

The optimum hydrolysis conditions for fetuin using 0.01 *M* HCl was determined by measuring the amount of sialic acid liberated after various time intervals up to 6 h.

3. Results and discussion

3.1. Hydrolysis

Sialic acids are similar to other 2-deoxy sugars in being very sensitive to treatment with mineral acids. The rate of decomposition rises with increasing acidity and temperature [22]. When heated in 0.1 *M* HCl at 100°C for 1 h, the sialic acids were judged to be unaffected using the resorcinol and Ehrlich reactions while a considerable decrease of the color was observed in the thiobarbituric acid reaction [23]. The sensitivity of sialic acids to mild acid treatment and their tendency to change from cyclic forms to the open chain form provided the impetus to investigate the hydrolysis time and the recovery of known amounts of the sialic acids using hydrolysis conditions described by Ogawa et al. [13], since these investigators used bovine vitronectin as a model. Fig. 2 shows that the sialic acids are completely released after 1 h at 80°C and that degradation does not start to occur until after 2 h. Approximately 10% degradation occurs between 2 and 4 h. This study was concluded after 6 h.

3.2. Chromatography

Different HPLC columns, which have been designed to operate with 100% water as the eluent, were evaluated for the determination of the sialic acids. Problems with reproducibility, peak broadening and peaks of interest eluting in the void volume were experienced. It was found necessary to store the column using the manufacturer's recommended solvent immediately after completing an analysis in order to avoid drastic changes in column retention

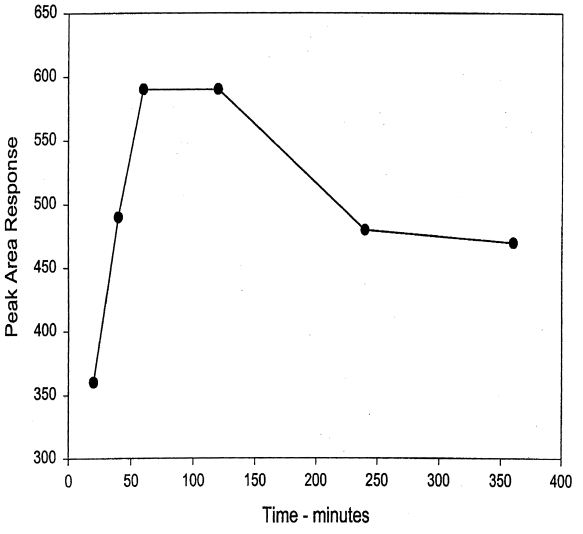

Fig. 2. Fetuin hydrolysis profile using 0.01 *M* HCl.

properties. The Ultra IBD column (Restek, Bellefonte, PA, USA), 5 μm, 150×4.6 mm, gave a different elution profile from the AquaSep column. With a flow-rate of 0.25 ml/min, the internal standard eluted at 5.88 min followed by NGNA at 7.23 min and NANA at 8.00 min. Hydrochloric acid (50 m*M*) works equally as well as a mobile phase but 0.1 *M* formic acid was chosen since it is not as corrosive on the electrospray interface.

3.3. Assay selectivity

Although asialofetuin [24] contains less than 0.5% total *N*-acetylneuraminic acid, it was not practical to analyze its hydrolysate as a blank since the LC–SIM-MS method is capable of detecting this amount of residual NANA. Non-hydrolyzed fetuin was used as a blank and the selected ion chromatograms are shown in Fig. 3. Fig. 4 shows the selected ion chromatograms of the 50 ng of NGNA and NANA standards with 50 ng I.S. and Fig. 5 shows the ion chromatograms for the hydrolyzed fetuin.

3.4. Hydrolysate recovery

The hydrolysis procedure was further evaluated by comparing the amounts recovered for standards spiked into 0.01 *M* HCl versus non-hydrolyzed

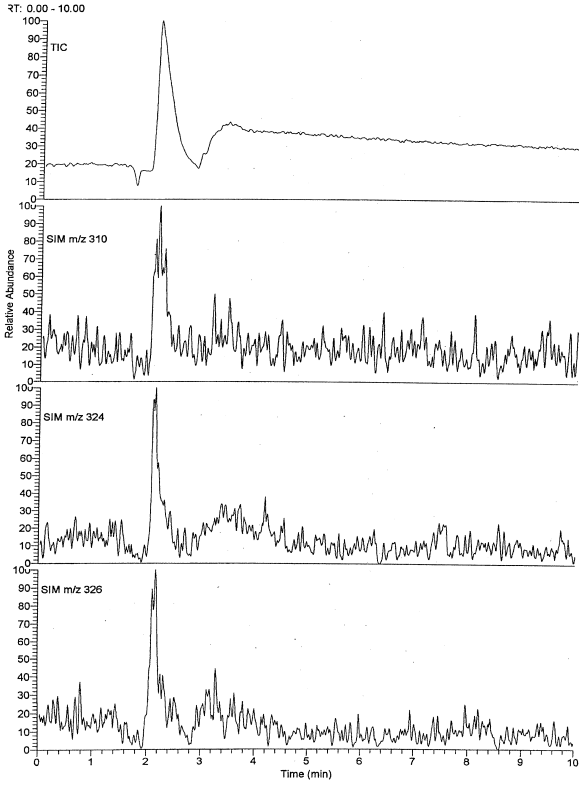

Fig. 3. Selected ion chromatogram for non-hydrolyzed fetuin which was used as a blank. RT, retention time in minutes.

standards. The hydrolysate recovery was conducted at all points on the calibration and the results are shown in Table 1. The overall mean recoveries of the analytes over the range of the standard curve were found to be 98%.

3.5. Linearity of calibration diagram

Linear regression calibration curves, which were established by plotting the peak area ratio of NGNA or NANA to the internal standard versus standard concentration, gave correlation coefficients of regression greater than 0.99 over the concentration range 5 to 100 μg/ml for each analyte. This represents 50 to 1000 ng on-column. The average peak area response ratios were used to obtain the calibration curve for NANA and NGNA. The slope, which was obtained using Sigma Plot 4.1, from the linear regression of the calibration curve was used to calculate the amounts of sialic acid in the fetuin hydrolysate: for

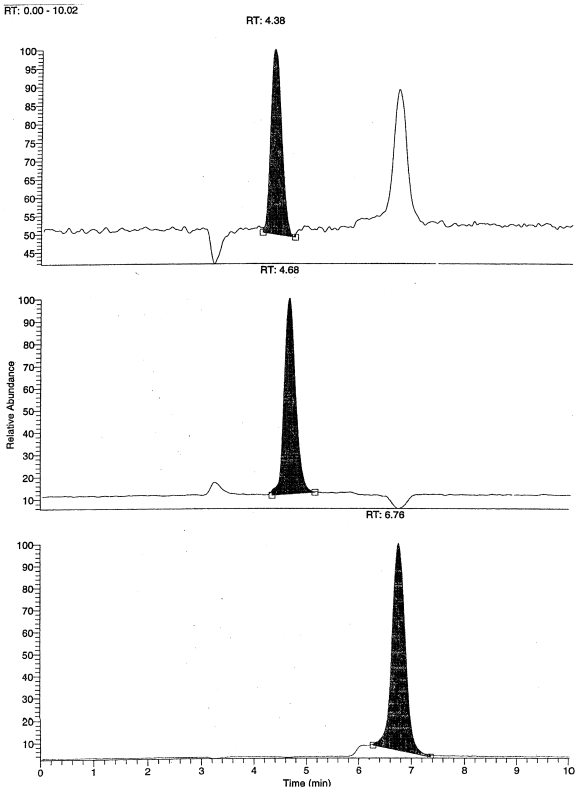

Fig. 4. Selected ion chromatograms for 50 ng standard on column Top, SIM m/z 326 NGNA; middle, SIM m/z 310 NANA; bottom, I.S. m/z 324.

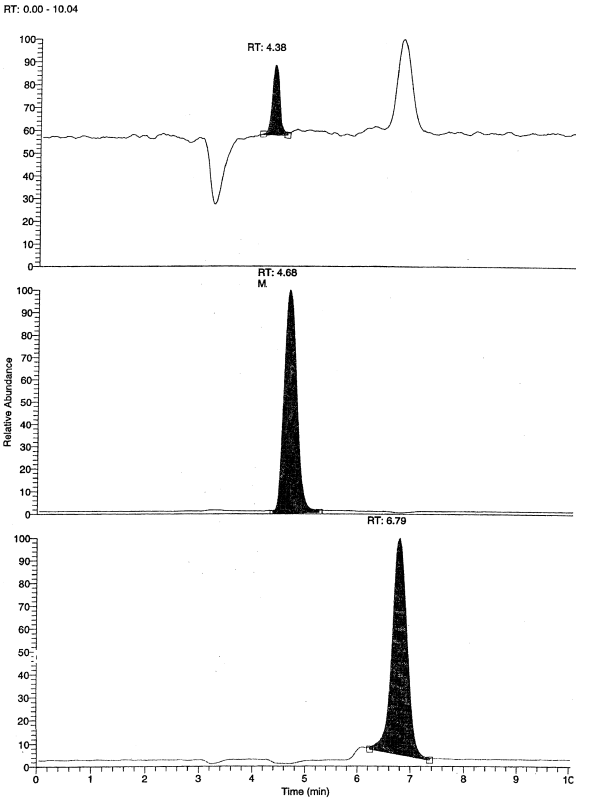

Fig. 5. Selected ion chromatogram for the fetuin hydrolysate sample. Top, SIM NGNA m/z 326; middle, NANA m/z 310; bottom, I.S. m/z 324.

NGNA $y = 0.0007x - 0.0028$, $R^2 = 0.9994$ and NANA $y = 0.0013x + 0.0786$, $R^2 = 0.9975$.

3.6. Assay precision and accuracy

The mean relative error [25] for NGNA was -0.91% and for NANA -2.83%. This was obtained by five measurements of the five different concentrations used to construct the calibration curve.

Replicate samples ($n = 5$) were analyzed to assess the within-day variability of the sialic acids. The mean concentrations as well as the relative standard deviations (RSDs) of the analyses (Table 2) were: NGNA, $\% = 0.198 \pm 0.005$, RSD $= 2.52\%$, $n = 5$ and NANA, $\% = 7.54 \pm 0.13$, RSD $= 1.72$, $n = 5$. The sialic acid expressed as *N*-acetylneuraminic acid has been reported by several investigators [26–29] and the value expressed in grams per 100 g of fetuin was summarized by Graham [29]. The previously reported sialic acid content ranges from 5.9 to 8.9%. Spiro et al. [26] found NANA to be 8.2% and 0.6% NGNA. The amount of free and total NANA in fetuin lot 117H7405 was 6.2%. Sigma determined this value using the thiobarbituric acid colorimetric procedure. The cause for disagreement between the Sigma value and the value reported in Table 2 is due to differences in the methods. In the colorimetric

Table 1
Spiked NGNA and NANA recovery after 1 h hydrolysis

Conc. (μg/ml)	Recovery (%)	NGNA RSD (%), $n = 3$	Recovery (%)	NANA RSD (%), $n = 3$
5	95.5	9.55	94.1	6.29
10	99.0	2.94	98.3	2.50
25	99.8	2.76	97.2	1.76
50	98.7	1.68	98.6	1.28
100	99.4	0.56	98.6	0.65

Table 2
Sialic acid content found in commercial fetuin, Sigma lot 117H7405

	NGNA (%)	NANA (%)
	0.20	7.39
	0.19	7.70
	0.20	7.45
	0.20	7.53
	0.20	7.63
Mean (%)	0.198	7.54
SD (%)	0.005	0.13
RSD (%)	2.52	1.72

procedure, the hydrolysis is very time dependent and ''times must be precisely observed''. Oxidation using periodate and incubation as well as subsequent color development has to be ''exactly'' timed. The levels of sialic acid found by LC–MS are within the range previously reported in the literature.

3.7. Limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by measurement of the baseline noise in the region that corresponded to the peaks of interest. For the purpose of this study, the LOD is equal to a 3:1 signal-to-noise level, while the LOQ corresponds to a 10:1 signal-to-noise ratio. NGNA's LOD was 1 ng and NANA's 0.5 ng. The LOQ for NGNA was 3.3 ng and the LOQ for NANA was 1.6 ng.

4. Conclusions

A method based on HPLC with MS detection using SIM for the determination of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid has been developed. The method is fast (overall time 10 min), sensitive, and selective for the quantitation of sialic acids and it has been successfully applied to determine the sialic acid content of fetuin. The sample procedure requires few manipulations and derivatization is not necessary.

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