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ANALYSIS OF N,O-ACYLATED NEURAMINIC ACIDS BY HIGH-PERFORMANCE LIQUID ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Analysis of different neuraminic acid derivatives can be performed within 10 min on a small column (40 × 4.6 mm) filled with the strong basic anion-exchange resin Aminex A-28 (particle size 9 μm), using 0.75 m*M* sodium sulphate solution as mobile phase. (For instance, the following retention times in seconds were obtained: N-acetylneuraminic acid, 361; N-glycolylneuraminic acid, 480; N-acetyl-9-mono-O-acetylneuraminic acid, 530). The effect of other anions such as chloride and acetate on the separation is described. The method allows determination of amounts of sialic acids as small as 6 ng (20 pmol). It is also used for the separation of sialyllactose isomers, sialyllactosamine and CMP-N-acetylneuraminic acid, and is compared with the behaviour of several neutral sugars and N-acylmannosamines. The method can be applied to follow enzymatic reactions of the sialic acid metabolism such as the actions of sialidase and N-acetylneuraminate lyase, and for preparation of individual sialic acids in small quantities. If combined with colorimetry, thin-layer or gas–liquid chromatography or with specific enzyme reactions, this method represents the fastest, most sensitive and specific technique available for the analysis of sialic acids.

INTRODUCTION

Sialic acids are derivatives of neuraminic acid with acetyl or glycolyl residues at the amino group and frequently with additional acetyl groups at O-4, O-7, O-8 and O-9 (ref. 1); sometimes lactyl groups are present at O-9 (ref. 2). The quantitative and qualitative analysis of sialic acids can be performed by colorimetry[3], fluorimetry[4], thin-layer and gas–liquid chromatography[3]. The structure of 23 naturally occurring sialic acids has been established by gas–liquid chromatography–mass spectrometry (GC–MS)[5], as well as by ^{1}H nuclear magnetic resonance spectroscopy[6]. These methods require purification of sialic acids before analysis and also derivatization for GC. The purification of sialic acids is time-consuming and may lead to loss of sialic acids and of O-acyl groups[3]. Therefore, rapid sialic acid determination, *e.g.*, from enzymatic reactions, is not possible, and a method for the direct analysis of sialic acids without prior purification and modification would be very useful.

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High-performance liquid chromatography (HPLC) has been described for the quantification of sialic acids as a whole on cation-exchange[7] and anion-exchange resins[8]. N-Acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and O-acetylated derivatives could be separated as borate complexes on a sugar analyzer using a strong basic anion-exchange resin[9].

Here we describe a simple, fast, accurate and very sensitive method for the separation of different natural derivatives of neuraminic acid by high-performance liquid anion-exchange chromatography. This method can be used for the quantification of sialic acids, separation of these substances on a preparative scale and for the study of enzymatic reactions.

MATERIALS AND METHODS

Chemicals and enzymes

N-Acetyl- and N-glycolylneuraminic acid and their derivatives O-acetylated at C-7, C-8 or C-9 were isolated from bovine submandibular gland glycoproteins[10], and 4-O-acetylated sialic acids were isolated from equine submandibular gland glycoproteins and erythrocytes[10]. Sialyllactose isomers and sialyllactosamine were prepared from bovine colostrum[11] and sialyllactose containing N-acetyl-9-mono-O-acetylneuraminic acid (Neu5,9Ac_2) was isolated from rat urine[12]. 2-Deoxy-2,3-dehydro-N-acetylneuraminic acid (5-acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid[13]) (Neu5Ac2en) was purchased from Boehringer (Mannheim, G.F.R.). Vibrio cholerae sialidase (E.C. 3.2.1.18) was obtained from Behringwerke (Marburg, G.F.R.) and Clostridium perfringens N-acetylneuraminate lyase (E.C. 4.1.3.3) from Sigma (München, G.F.R.). Standard monosaccharides were purchased from Koch-Light (Colnbrook, Great Britain). Silica gel 60 (0.2 mm) and cellulose thin-layer (0.1 mm) plates, solvents and chemicals of analytical grade were products of E. Merck (Darmstadt, G.F.R.). Anion-exchange resin (Aminex A-28, particle size 9 μm) was purchased from Bio-Rad (München, G.F.R.). Doubly distilled water was used throughout the experiments.

High-performance liquid chromatography (HPLC)

HPLC was performed on a Spectra-Physics SP 8000 apparatus equipped with a photometer (Spectroflow monitor SF 770, Schoeffel Instrument Corp.) and a SP 4000 integrator for automatic calculation of peak areas. Chromatography was performed in a small column of stainless steel (40 × 4.6 mm I.D.) containing Aminex A-28, usually at room temperature. Before filling, the resin was converted into the desired ionic form. The slurry was packed into the column in the same solution or buffer in which the chromatography was performed; this is necessary to avoid excessive back pressure. After a few hundred runs, the resin was taken out and regenerated by treatment with 3 *M* HCl at 50°C for 10 min.

For analysis, samples usually containing 0.1–10 μg of sialic acids dissolved in water or buffers (in some cases taken from enzymatic incubation mixtures) were applied to the column, which contained a 10-μl sample loop. In some experiments, O-acetylated sialic acids were O-deacetylated with 0.1 *M* NaOH, as described in ref. 4, before application to the column to identify whether these sialic acids were derivatives of Neu5Ac or Neu5Gc.

Sialic acids were eluted isocratically by 0.75 m*M* sodium sulphate or 1 m*M* sodium chloride solution, or 40 m*M* sodium acetate buffer, pH 5.5. Sometimes a gradient from 0 to 0.75 m*M* sodium sulphate was used. The solutions were degassed with helium. The flow-rates during the chromatography were 0.3–1 ml/min at 5–30 bar. Eluted sialic acids were monitored at wavelengths between 195 and 215 nm; detector sensitivity was 0.04 a.u.f.s. and attenuation varied between 5 and 40 mV.

Analysis of sialic acids after HPLC

Sialic acids eluted from the HPLC column were further analyzed by microadaptations of the periodic acid–thiobarbituric acid and the orcinol–Fe^{3+}–HCl assays[3], and by thin-layer chromatography on cellulose. For these purposes, corresponding sialic acid fractions from several runs were collected and salts were removed from the mobile phase by means of cation- and anion-exchange chromatography, as described in ref. 3. The cellulose plates were prerun in 0.1 *M* HCl and dried before application and development of the sialic acids in *n*-butanol–*n*-propanol–0.1 *M* HCl (1:2:1)[3]. For the separation of sialyllactose isomers silica gel-60 thin-layer plates were used in ethanol–*n*-butanol–pyridine–water–acetic acid (100:10:10:30:3)[11]. Sialic acid spots were visualized by the orcinol–Fe^{3+}–HCl spray reagent[3].

Enzymatic reactions

Reactions of Vibrio cholerae sialidase with sialyllactose isomers and α_1-acid glycoprotein were carried out in 50 m*M* sodium acetate buffer, pH 5.5, containing 154 m*M* sodium chloride and 9 m*M* calcium chloride[3]. The incubation mixtures (100 μl) contained about 5–10 μg of glycosidically bound sialic acid and 2–10 mU sialidase. After different incubation times at 37°C, samples of 10 μl were applied to the column for determination of the amount of liberated sialic acid.

Sialic acids were cleaved by N-acetylneuraminate lyase in 20 m*M* sodium phosphate buffer, pH 7.2 (ref. 3). The incubation mixtures (100 μl) contained about 2–10 μg sialic acid and 5–10 mU enzyme. After different times at 37°C, samples of 10 μl were withdrawn and applied to the column to estimate the amount of residual sialic acid and the formation of acylmannosamine.

RESULTS AND DISCUSSION

The retention times of different N,O-acylated derivatives of neuraminic acid including cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) and various sialyloligosaccharides are shown in Table I, and are compared with some neutral sugars and N-acylmannosamines. To illustrate the extent of separation of different sialic acids the elution profiles of sialic acid mixtures isolated from bovine submandibular gland glycoproteins and from equine erythrocyte membranes are shown in Fig. 1. A baseline separation of Neu5Ac and Neu5Gc, which are the most common sialic acids in nature, can be achieved within 5 min in an isocratic 0.75 m*M* sodium sulphate solution as mobile phase and at a flow-rate of 1 ml/min. However, under these conditions the column cannot be used for a longer time, as after some runs problems of back pressure arise. It is therefore better to operate at a flow-rate of 0.5 ml/min allowing separation within 10 min (Fig. 2). The flow-rate does not significantly influence the quality of separation.

TABLE I

RETENTION TIMES OF DIFFERENT SIALIC ACIDS, CMP-Neu5Ac, VARIOUS SIALYLOLIGOSACCHARIDES AND A FEW OTHER MONOSACCHARIDES ON ANION-EXCHANGE HPLC

Sodium sulphate (0.75 m*M*) was taken as mobile phase. The flow-rate was 0.5 ml/min at 15 bar. For further details see Materials and methods.

Compound	*Retention time (sec)*
Glucose	39
Galactose	39
Mannose	39
N-Acetylmannosamine	43
N-Glycolylmannosamine	49
Di-N-acetylneuraminyllactose	99
CMP-N-acetylneuraminic acid (CMP-Neu5Ac)	105
N-Acetylneuraminyl-α(2-6)-N-acetyllactosamine	115
N-Acetylneuraminyl-α(2-6)-lactose	135
N-Acetylneuraminyl-α(2-3)-lactose	173
N-Acetyl-9-mono-O-acetylneuraminyl-α(2-3)-lactose	205
N-Acetylneuraminic acid (Neu5Ac)	361
N-Glycolylneuraminic acid (Neu5Gc)	480
N-Acetyl-4-mono-O-acetylneuraminic acid ($Neu4,5Ac_2$)	510
N-Acetyl-9-mono-O-acetylneuraminic acid ($Neu5,9Ac_2$)	530
2-Deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en)	605
N-Glycolyl-4-mono-O-acetylneuraminic (Neu4Ac5Gc)	610
N-Glycolyl-9-mono-O-acetylneuraminic acid (Neu9Ac5Gc)	615
N-Acetyl-7,9-di-O-acetylneuraminic acid ($Neu5,7,9Ac_3$)	636
N-Glycolyl-7,9-di-O-acetylneuraminic acid ($Neu7,9Ac_2$5Gc)	730
N-Acetyl-7,8,9-tri-O-acetylneuraminic acid ($Neu5,7,8,9Ac_4$)	740
N-Glycolyl-7,8,9-tri-O-acetylneuraminic acid ($Neu7,8,9Ac_3$5Gc)	910

By this method, the mono-, di- and tri-O-acetylated sialic acids can be well separated from each other and from the non-O-acetylated sialic acids, as shown in Table I. Exceptions are mono-O-acetylated Neu5Ac on the one hand and Neu5Gc on the other, having O-acetyl groups at different positions, which cannot be clearly distinguished. For example, $Neu4,5Ac_2$ and $Neu5,9Ac_2$ elute after almost the same time (Table I), as do the pair Neu4Ac5Gc and Neu9Ac5Gc. In contrast, Neu5Ac and Neu5Gc, both O-acetylated at C-9, can be well separated.

The values given in Table I depend on the ionic strength and the nature of anions present in the mobile phase. Sodium sulphate (0.75 m*M*) proved to be most suitable for the separation of sialic acids. On further increasing the concentration of sodium sulphate the separation becomes poorer, but sialic acids elute faster. Thus, 2 m*M* sodium sulphate can be applied for the analysis of enzymatic reactions (see below), where usually only one sialic acid is present and a fast identification is required. The replacement of sodium sulphate by sodium chloride or sodium acetate increases the retention times in the order $SO_4^{2-} < Cl^- < CH_3COO^-$. For an optimal separation, higher concentrations of chloride and acetate than of sulphate are required. Examples are shown in Table II. In contrast to the sodium acetate buffer, the separation of Neu5Gc from mono-O-acetylated Neu5Ac is poor in the sodium sulphate system (Table II). As, however, Neu5Ac and Neu5Gc cannot be well separated

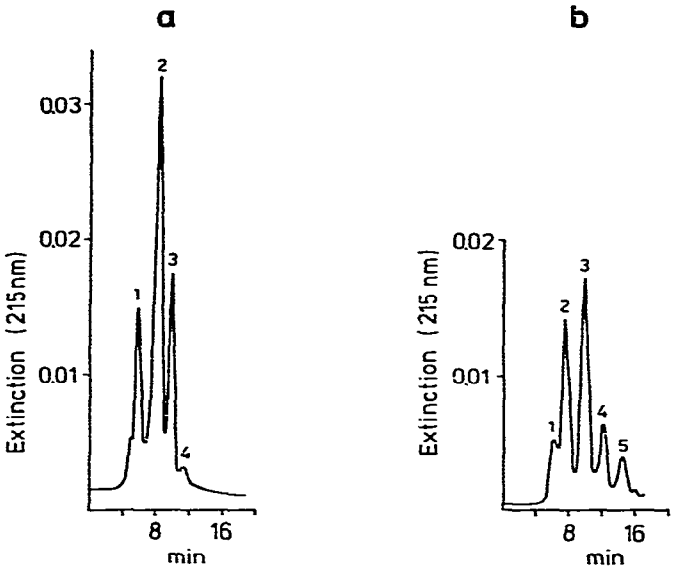

Fig. 1. Elution profiles from anion-exchange HPLC of sialic acid mixtures isolated by mild acid hydrolysis[3,10] from bovine submandibular gland glycoproteins (a) and equine erythrocyte membranes (b). The mobile phase was 0.75 m*M* sodium sulphate and the flow-rate 0.5 ml/min at 15 bar. Sialic acids were monitored at 215 nm by 0.04 a.u.f.s. and attenuation 5 mV. Peaks: a, 1 = Neu5Ac; 2 = Neu5,9Ac$_2$ containing a small amount of Neu5Gc; 3 = Neu5,7,9Ac$_3$; 4 = Neu5,7,8,9Ac$_4$; b, 1 = Neu5Ac; 2 = Neu5Gc; 3 = Neu4Ac5Gc; 4 = Neu4,9Ac$_2$5Gc; 5 = Neu4,7,9Ac$_3$5Gc.

in sodium acetate buffer, in contrast to the sodium sulphate system, combination of both systems for the analysis of different sialic acids is recommended.

Separation of sialic acids in the sodium sulphate system can be improved by using a linear gradient of 0–0.75 m*M* sodium sulphate solution (10 ml; speed 0.5 ml/min). Although this is a relatively time-consuming separation for analytical purposes, it can successfully be used for the preparative separation of sialic acids, where 50–60 μg can be fractionated in each run using a column of the size described.

It is known for ion-exchange chromatography that increasing the tempera-

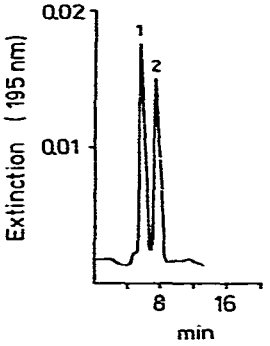

Fig. 2. Elution profile of a Neu5Ac (1) and Neu5Gc (2) mixture (each 200 ng) from anion-exchange HPLC. The mobile phase was 0.75 m*M* sodium sulphate and the flow-rate 0.5 ml/min at 15 bar. The sialic acids were monitored at 195 nm by 0.04 a.u.f.s. and attenuation 10 mV.

TABLE II

RETENTION TIMES OF SOME SIALIC ACIDS IN DIFFERENT MOBILE PHASES

Mobile phases: a, 0.75 m*M* sodium sulphate solution; b, 1 m*M* sodium chloride solution; c, 40 m*M* sodium acetate buffer, pH 5.5. The flow-rate was 0.5 ml/min at 15 bar. For further details see Materials and methods.

Sialic acids	*Retention times (sec)*		
	a	*b*	*c*
Neu5Ac	360	700	930
Neu5Gc	480	780	990
Neu5,9Ac_2	530	900	1220
Neu5Ac2en	605	970	1530

ture[14] results in better separation. This was also observed with sialic acids. For example, the theoretical plates for Neu5Ac can be calculated to be 630 at 20°C and 1298 at 70°C in the column used in these studies, leading to a better separation of Neu5Ac and Neu5Gc at the higher temperature (retention times in sec at 20°C: Neu5Ac, 361; Neu5Gc, 480; and at 70°C: Neu5Ac, 310; Neu5Gc, 512). In spite of these observations, only room temperature was used routinely in these studies, as otherwise some O-deacetylation of sialic acid occurs.

The relationship between peak areas and wavelengths of constant amounts of Neu5Ac is indicated in Fig. 3, showing that this sialic acid can be detected with 15 times higher sensitivity at 195 nm than at 215 nm. Neu5Gc and O-acetylated sialic acids behave similarly. The absorbancy of Neu5Ac2en is less influenced than that of the saturated sialic acids by the wavelength (Fig. 3). On a molar basis, at 215 nm the sensitivity of Neu5Ac2en is about 30 times higher than that of Neu5Ac or Neu5Gc, probably due to the conjugated double bonds. If sufficient amounts of sialic acid were present in the samples, the eluates were monitored at 215 nm, as substances other than sialic acids may interfere less at this wavelength than at 195 nm.

A standard curve for the determination of Neu5Ac is shown in Fig. 4, indicating that a minimum amount of 30 ng (0.1 nmol) of this sialic acid can be measured. Similar values were obtained for Neu5Gc and for O-acetylated derivatives. Chosing the more sensitive absorption conditions at 195 nm, 0.01 a.u.f.s. and attenuation 2.5 mV, a minimum amount of 6 ng (20 pmol) of sialic acids can be determined. According to the observations described in the foregoing paragraph, an appreciably smaller amount of Neu5Ac2en can still be identified. The error range in the calculation of peak areas is less than 1% and the variation of the peak positions is less than 0.5%.

The method used for the identification of sialic acids by monitoring at 215 or 195 nm is not specific for sialic acids, and substances other than sialic acids present as impurities in the sialic acid preparations may interfere with the analysis. The specificity may be increased by using post-column reactions specific for sialic acids, *e.g.*, periodic acid–thiobarbituric acid[8] or orcinol–Fe^{3+}–HCl assays, or by analysis of the eluted sialic acids by thin-layer or gas–liquid chromatography. When applying these analytical tools, a recovery of the total amount of sialic acids eluted from the column was obtained. O-Acetylated sialic acids were rechromatographed on HPLC after O-deacetylation to show whether they were derivatives of Neu5Ac or Neu5Gc.

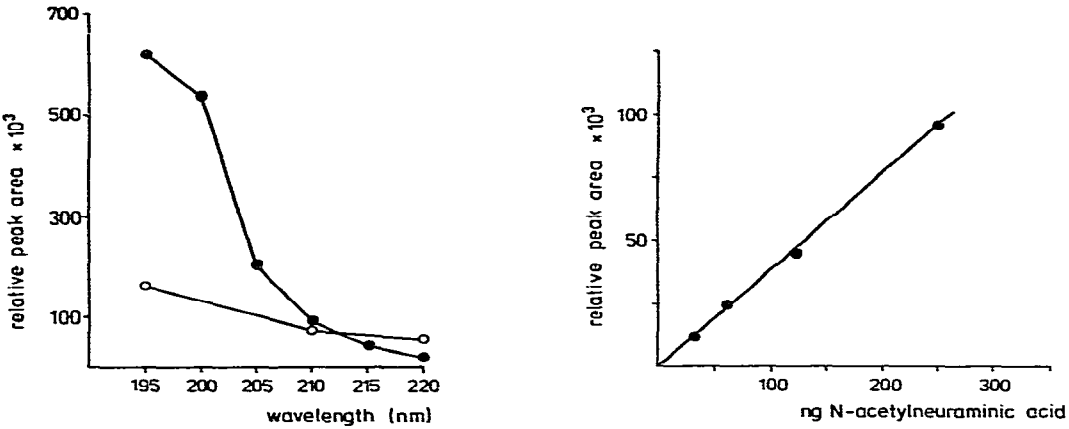

Fig. 3. Dependence of peak areas of 2 μg Neu5Ac (●) and 60 ng Neu5Ac2en (○) on the wavelength (nm) by which sialic acids were detected on anion-exchange HPLC. Sodium sulphate solution (0.75 m*M*) was taken as mobile phase. The flow-rate was 0.5 ml/min at 15 bar.

Fig. 4. Calibration curve for Neu5Ac on anion-exchange HPLC by using 0.75 m*M* sodium sulphate as mobile phase. The flow-rate was 0.5 ml/min at 15 bar. Neu5Ac was monitored at 195 nm.

The most specific determination of sialic acids is possible by the use of N-acetylneuraminate lyase. This enzyme cleaves sialic acids into acylmannosamines and pyruvic acid[3,15]. Therefore, samples containing sialic acids were applied to the HPLC column before and after incubation with this enzyme, and the decrease in the peak area of sialic acids was used as proof for the presence of these substances. In addition, the formation of acylmannosamines can be monitored in the same run (for the retention times of N-acetyl- and N-glycolylmannosamine see Table I).

This method can also be used for the rapid determination of the activity of N-acetylneuraminate lyase. This enzyme assay is very sensitive and more specific when compared to other tests, for example, determination of formed pyruvic acid by lactate dehydrogenase[16]. In Fig. 5 the rates of cleavage of Neu5Ac and Neu5Gc by N-acetylneuraminate lyase are compared. Neu5Ac was cleaved at a rate 30–35% higher than Neu5Gc, thus confirming earlier results obtained with other methods[15–17].

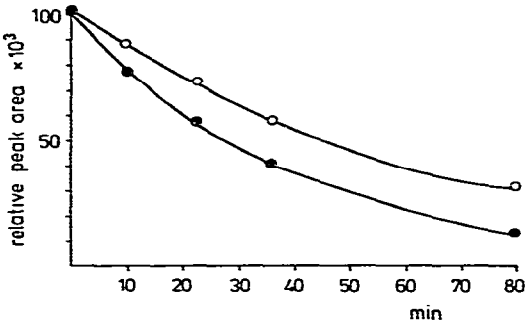

Fig. 5. Cleavage of Neu5Ac (●) and Neu5Gc (○) by N-acetylneuraminate lyase. Sialic acids were analyzed by anion-exchange HPLC, using 0.75 m*M* sodium sulphate as mobile phase at a flow-rate of 0.5 ml/min and 15 bar and monitoring the eluate at 215 nm.

N-Acetylneuraminyllactose isomers [α(2-3) and α(2-6)], N-acetylneuraminyl-α(2-6)-N-acetyllactosamine, N-acetyl-9-mono-O-acetylneuraminyl-α(2-3)-lactose and di-N-acetylneuraminyllactose can also be separated by the HPLC method described. The quality of separation is much better in the acetate buffer than in the sulphate solution, as shown in Table III. The sialyllactose isomers have been used as models for the study by HPLC of the action of sialidase. The rate of hydrolysis of 2-3 or 2-6 isomers of sialyllactose by V. cholerae sialidase was determined either by the change in the peak area of sialyllactose or by the formation of free Neu5Ac. The 2-6 isomer was shown to be hydrolyzed at a rate 60% lower when compared to the 2-3 isomer, as was found earlier by other methods[18]. In place of sialyllactose, α_1-acid glycoprotein or other substrates can be used for the determination of sialidase activity by the HPLC technique. Samples from the incubation medium can be directly applied to the column. The advantages of this HPLC method over the previous methods for the determination of sialidase activity are the rapidity and simplicity of analysis, thus allowing kinetic measurements of the activity of this enzyme.

TABLE III

RETENTION TIMES OF SIALYLOLIGOSACCHARIDES ON ANION-EXCHANGE HPLC

Mobile phases: a, 0.75 m*M* sodium sulphate solution; b, 40 m*M* sodium acetate buffer, pH 5.5. The flow-rate was 0.5 ml/min at 15 bar. For further details see Materials and methods.

Sialyloligosaccharide	*Retention times (sec)*	
	a	*b*
Di-N-acetylneuraminyllactose	99	158
N-Acetylneuraminyl-α(2-6)-lactose	135	216
N-Acetylneuraminyl-α(2-3)-lactose	173	320

The application of the Aminex A-28 phase described here appears to have advantages over the silica gel amino-phase, which was used earlier for the separation of sialyllactose isomers[19]. The silica gel amino-phase has been described to be not as stable[20] as anion-exchange resins and cannot be used as frequently or be regenerated, the latter being necessary if the solutions contain proteins.

In conclusion, sialic acids can be both separated and quantified within a very short time. The method described is the fastest and most sensitive technique known for the analysis of sialic acids, requiring only 6 ng of substance. A further advantage is that the sialic acids can be analyzed without prior extensive purification and derivatization. The specificity of the method may be increased by subjecting the sialic acids, recovered quantitatively from the column, to further analyses. These facts prove useful for kinetic measurements of enzymatic reactions of sialic acid metabolism; two examples have been described. In addition, this HPLC technique may be extended to preparative purposes.

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