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Determination of N-acetyl- and N-glycolylneuraminic acids in glycoconjugates by reversed-phase high-performance liquid chromatography with ultraviolet detection

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SUMMARY

A rapid and sensitive method for the determination of sialic acids is described. The measurement is based on isocratic high-performance liquid chromatography, whereby N-acetylneuraminic and N-glycolylneuraminic acids are separated. The total amounts of these acids can be determined after hydrolysis and per-O-benzoylation. The conditions for hydrolysis and derivatization were optimized for measurement of these sialic acids in glycoconjugates.

The benzoyl derivatives were chromatographed on a reversed-phase column with 67% (v/v) aqueous acetonitrile and the eluted peaks were monitored by UV detection. The method allows the determination of picomole amounts. the reaction was shown to give linear calibration graphs over the entire range tested, *i.e.*, up to 160 nmol (50 μg) of each of the sialic acids.

INTRODUCTION

Sialic acids are widely distributed in nature, mainly as oligosaccharide components of glycoproteins and gangliosides, but also in glycosaminoglycans[1,2]. They constitute a family of neuraminic acid (5-amino-3,5-dideoxy-D-nonulosonic acid) derivatives. These compounds are divided into two major groups, depending on the

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nature of their N-substitution. Most naturally occurring forms are N-acetylated, whereas in others the amino group carries a glycolyl group, the relative proportions of these forms differing in various tissues in different species. Often these structures are also acetylated on the hydroxyl groups. Such O-acetylation may be found at one or more of any of the available hydroxyl groups, *i.e.*, at O-4, O-7, O-8 and/or O-9[3,4]. The functions of the different N-acyl and O-acetyl groups are not completely understood, but the variability of these constituents is of importance, *e.g.*, in enzymatic reactions[5,6].

Spectrophotometric[7–13] and enzymatic [14] reactions, thin-layer chromatography[13] and gas chromatography[13,15–17] have all been used to determine sialic acids. Gas–liquid chromatography[18–20] and high-performance liquid chromatography (HPLC)[21] combined with mass spectrometry and also ^{1}H NMR spectrometry[22,23] have been applied to the analysis and further elucidation of the structures of more than 30 sialic acids with different acetylation patterns.

Liquid column chromatography, including HPLC, has also been used for the quantification of sialic acids. For such determinations both cation-[24] and anion-exchange[4,12,18,25–29] or anion-exclusion[30] resins have been employed. With such a resin it is possible to separate N-glycolylneuraminic acid (Neu5Gc), N-acetylneuraminic acid (Neu5Ac) and several O-acetylated derivatives of Neu5Ac[6,26]. Reversed-phase resins can also be used to obtain information regarding the content of Neu5Ac[31]. The sensitivity of these methods is limited mainly by the detection mode used. Thus low UV absorbance can be used, but the performance will be improved with postcolumn reactions[30,32] or by the use of precolumn derivatization, when the eluates may be recorded at higher wavelengths. One such method, which involves labelling with a fluorochrome, permits the separation and determination of different sialic acids in small amounts[33].

We have reported[34] that uronic acids and neutral monosaccharides could be determined in picomole amounts by per-O-benzoylation and subsequent HPLC separation. In the same way, hydroxyl groups of sialic acids can be per-O-benzoylated to form stable and strongly UV-absorbing derivatives suitable for the simpler and more accessible UV detection at a higher wavelength.

The liberation of sialic acids from glycoconjugates, which is necessary for their separation and determination, is most often achieved by means of acid hydrolysis[4,12,13,33,35] or enzymatic digestions[6,28]. The latter means of obtaining free sialic acids is dependent on several factors that are difficult to control in biological preparations. Thus O-acetylation, the occurrence of the N-glycolyl group and the conformation of the glycosidic linkage all interfere considerably with the enzymatic activity.

Acid hydrolysis is an effective means of cleaving the glycosidic bonds of the sialic acids. With most acids this liberation is followed by simultaneous removal of O-acetyl groups, whereas the N-acyl linkages are more stable. Most sialic acids can thus be recovered in their two basic forms, Neu5Ac and Neu5Gc. The hydrolytic procedure is always associated, however, with some destruction of these carbohydrates. If such a method is to be used for the release of sialic acids, it is important to check the possible influence on the subsequent derivatization and separation.

This study was therefore undertaken with the aim of finding suitable conditions for the per-O-benzoylation and subsequent chromatographic determination of Neu5Ac and Neu5Gc by HPLC with UV detection. A additional purpose was to find

optimum conditions for the release of these substances from glycoprotein samples, so that the separation procedure could be used for the determination of their contents of Neu5Ac and Neu5Gc.

EXPERIMENTAL

Chemicals

N-Acetylneuraminic and N-glycolylneuraminic acids and N-acetylneuraminyl-α((2,3)-lactose from human milk were purchased from Sigma (St Louis, MO, U.S.A.). Fetuin (type III) from foetal calf serum, mucin (type I-S) from bovine submaxillary glands and human α_1-acid glycoprotein (orosomucoid) were also obtained from Sigma. All other chemicals were of analytical-reagent grade.

Sample preparation

Sialic acids were liberated from glycoproteins by acid hydrolysis. This was performed in 250 μl of 25 m*M* hydrochloric acid or trifluoroacetic acid (TFA) for 2 h at 80°C in screw-capped polypropylene microtubes and the hydrolysates were subsequently lyophilized. As a control of sialic acid destruction, known amounts of Neu5Ac and Neu5Gc were kept under similar hydrolytic conditions. To test the hydrolytic conditions, experiments with various concentrations (up to 100 m*M*) of these acids were also performed. In addition, the effects of 25 m*M* sulphuric acid[33] and 2 *M* acetic acid[35] were tested.

Derivatization procedure

Per-O-benzoylation was performed by a micromodification of the methods described by Daniel *et al.*[36] and Jentoft[31]. To the dry hydrolysate 100 μl of benzoylation mixture [10% (w/v) benzoic anhydride–5% (w/v) *p*-dimethylaminopyridine in pyridine] were added. The mixture was heated at 80°C for 20 min. The conditions for this reaction were also tested by varying the reaction time and temperature. The reaction was terminated by adding 9 volumes of water and shaking vigorously on a vortex mixer. For complete destruction of the remaining benzoic anhydride, the mixture obtained was heated for a further 10 min at 80°C.

Excess of reagents and underbenzoylated derivatives were then removed by passing the mixture through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.), but eluting directly with 5 ml of water, omitting the aqueous pyridine purification step[36]. The sialic acid derivatives were eluted with only 5 ml of acetonitrile. After evaporation, the dry residue was dissolved in 1 ml of acetonitrile and centrifuged at 10 000 *g* for 5 min in a Beckman Microfuge. Aliquots of up to 20 μl were then injected into the chromatograph. If larger volumes were to be injected, the dried derivatives were preferably dissolved in 67% (v/v) aqueous acetonitrile.

High-performance liquid chromatography

The samples were added to the HPLC column by a loop injector. The separation was performed in a 250 mm × 4.6 mm I.D. column of Supelcosil LC-18 (particle size 5 μm) (Supelco, Bellfonte, PA, U.S.A.), equipped with an RP-18 guard column (30 mm × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.). The samples were eluted with 67% (v/v) aqueous acetonitrile at room temperature and at a flow-

rate of 1.5 ml/min. The eluted peaks were recorded at 231 nm, using a Beckman (Berkeley, CA, U.S.A.) Model 165 variable-wavelength UV detector.

RESULTS AND DISCUSSION

The per-O-benzoylated derivates of Neu5AC and Neu5Gc gave rise to only one major peak each (Fig. 1). These two peaks were widely separated in the LC-18 column when the samples were eluted with 67% (v/v) aqueous acetonitrile, the retention being 6.9 and 14.8 column volumes (9.2 and 19.5 min), respectively. It is therefore possible to separate these two sialic acids from each other also with higher acetonitrile concentrations, thereby increasing the sensitivity and reducing the elution times. Close to the Neu5AC, however, there are some minor peaks that possibly correspond to underbenzoylated derivatives or perhaps residual benzoic anhydride. In order to avoid coelution of Neu5Ac with these unidentified peaks, it is therefore of advantage to use a mobile phase with higher polarity and consequently longer retention times.

The greater retention of Neu5Gc is compatible with the extra site for benzoylation provided by the glycolyl substituent. Hence it seems as if the Neu5Gc derivative carries one benzoyl group on each of its six hydroxyl residues. This form of sialic acid, however, is more polar than the neutral hexoses because of the presence of the carboxyl and N-glycolyl groups and thus it elutes well before the hexoses. The

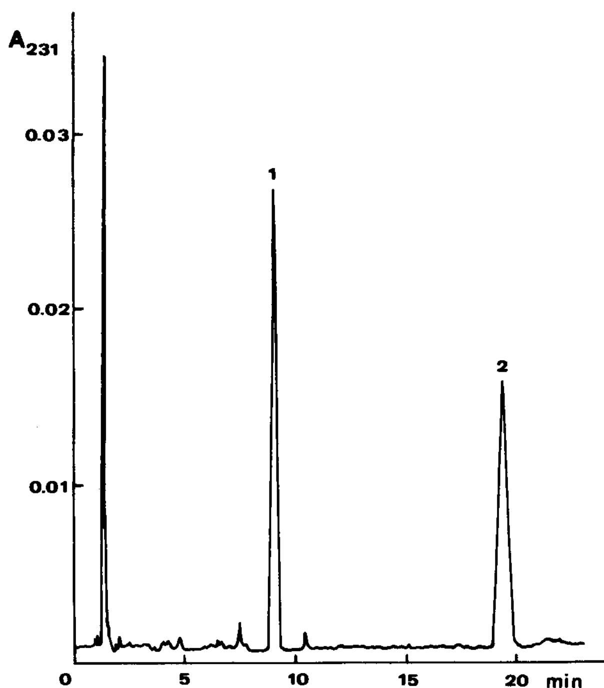

Fig. 1. Chromatogram of the per-O-benzoylated derivatives of (1) Neu5Ac and (2) Neu5Gc. The reversed-phase column (Supelcosil LC-18) was eluted with 67% (v/v) aqueous acetonitrile at 1.5 ml/min and the peaks were recorded at 231 nm.

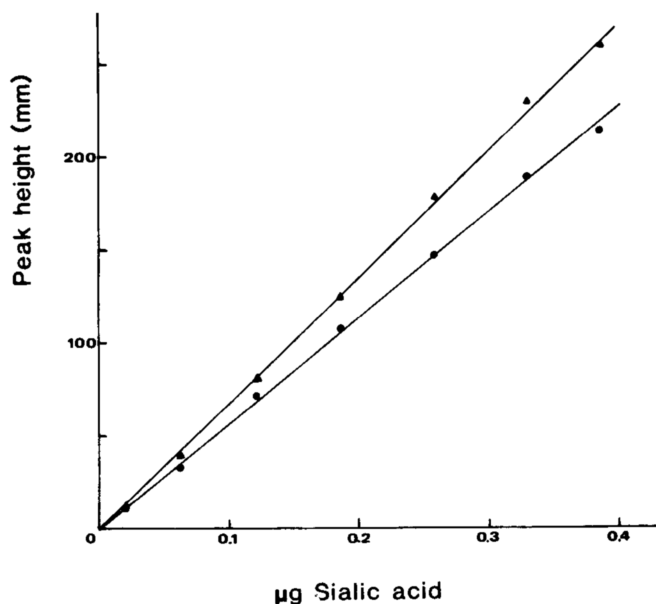

Fig. 2. Calibration graphs obtained with various amounts of (●) Neu5Ac and (▲) Neu5Gc taken for per-O-benzoylation and subsequent chromatography.

retention of Neu5Gc is, in fact, close to that of glucosamine. The anomeric peaks of this hexosamine elute 11.6 and 13.8 column volumes after the front, and will be separated from Neu5Gc with a column performance exceeding 7000 theoretical bottom plates. Galactosamine is recovered in two peaks after 11.6 and 12.3 column volumes, *i.e.*, well before Neu5Gc. When isolated from biological glycoconjugates under the present mild hydrolytic conditions, however, most hexosamines remain N-acetylated and they are therefore eluted much earlier than any of the sialic acids[34].

As with neutral sugars, the per-O-benzoylation will make the carbohydrates highly UV absorbent, allowing their determination in very small amounts. The sensitivity and linearity were both tested with the use of standard sialic acid mixtures (Fig. 2). The reaction with these preparations was linear up to 160 nmol, *i.e.*, when 50 μg of the sialic acid were injected into the column. With this amount the absorbance was close to 2, and it therefore seems as if the range for the analysis is limited mainly by the performance of the detector. The precision of the method was determined by six repeated determinations of both sialic acids. When 15 nmol of each acid were measured, the relative standard deviation was 2.8% for Neu5Ac and 2.6% for Neu5Gc, and with 6 nmol the corresponding figures were 3.1% and 2.9% respectively. The detection limit, expressed as twice the baseline noise, corresponded to 30 pmol (10 ng) of Neu5Ac injected, and as little as 600 pmol (0.2 μg) can thus be determined within a 95% confidence interval. The peak for Neu5Gc was approximately 20% higher than for Neu5Ac, in spite of the much longer retention of the N-glycolyl compound. The differences in peak areas are in good agreement with the assumption that the Neu5Gc derivative contains one more benzoyl residue than Neu5Ac.

With this separation as the measuring device, we studied the conditions for the

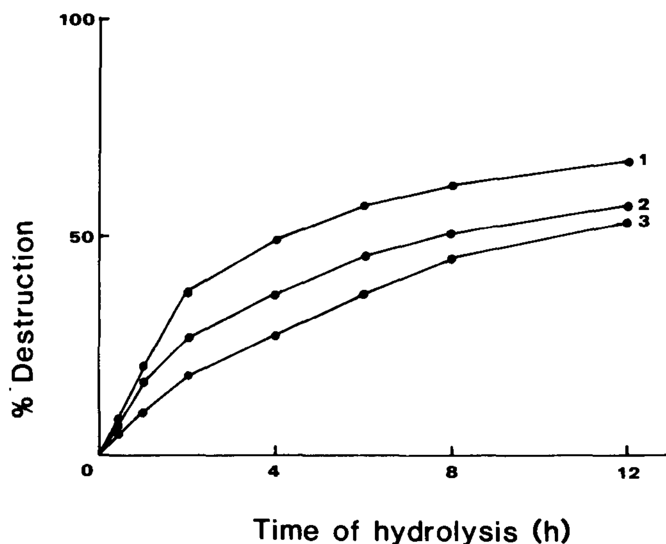

Fig. 3. Destructive effect of the hydrolytic conditions on Neu5Ac with use of (1) 100, (2) 50 and (3) 25 m*M* hydrochloric acid. Similar curves were obtained when the hydrolysis was performed with TFA and also when the effect was tested on Neu5Gc.

derivatization reactions and for the hydrolytic release of sialic acids from glycoconjugates. An increase in temperature from 37 to 80°C during the benzoylation reaction did not significantly affect the reaction yield. At 80°C the rate of the reaction was higher and it was completed within 20 min, whereas at 37°C a reaction time of 90 min was needed. The additional heating of the mixture for 10 min after the termination of the reaction significantly decreased the interference of early eluting peaks, which were also present in blank preparations and thus probably represented the benzoic anhydride.

The effect of the hydrolytic conditions on the Neu5Ac and Neu5Gc standards was studied with various acids as decribed above and at different concentrations. As shown in Fig. 3, there was some destruction depending on the concentration of the acid and the time of hydrolysis. The positions of the eluted peaks were unaltered, and hence there were no signs indicating specific removal of N-acyl groups by any of the acids tested. As shown previously[33], the hydrolysis with 25 m*M* sulphuric acid was associated with only minor losses. These hydrolysates were difficult to lyophilize, however, and it therefore does not seem practical to use this acid. The losses observed with 25 m*M* hydrochloric acid, 25 m*M* TFA and 2 *M* acetic acid were all similar. Acetic acid hydrolysis may, however, leave more O-acetyl groups unhydrolysed, which would then interfere with the subsequent chromatography. Hydrolysis with 25 m*M* hydrochloric acid or TFA seem to be the best of the tested alternatives when studying sialic acids in glycoconjugates. Although hydrolysis with these acids for 2 h will result in losses of *ca.* 20%, this time was preferred to ensure a complete hydrolysis of the glycoconjugate samples (see below).

It has been claimed that the time necessary for the hydrolysis of the sialic acid glycosidic linkages will be dependent on the glycoconjugate[33]. Our results with use of

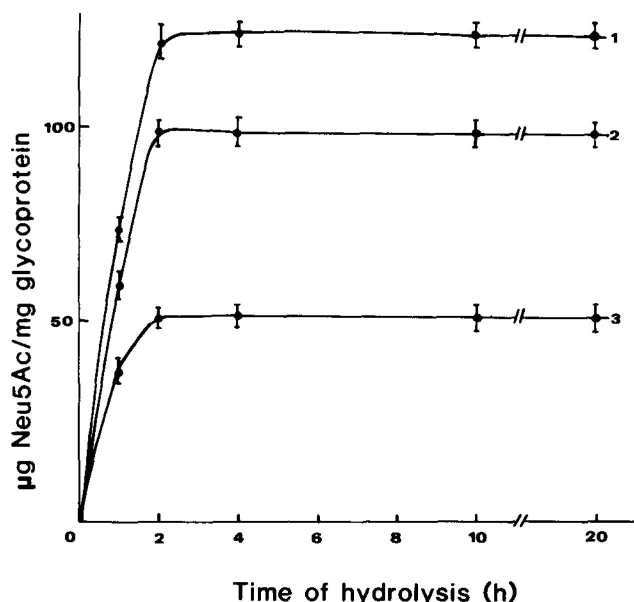

Fig. 4. Rate of liberation of Neu5Ac from glycoproteins when hydrolysis was performed with 25 m*M* hydrochloric acid at 80°C. Similar curves were obtained with 25 m*M* TFA. The amount of liberated sialic acid was determined by comparison with standard sialic acid mixtures treated under the same conditions. 1 = Bovine submaxillary mucin type I-S; 2 = human α_1-acid glycoprotein; 3 = foetal calf serum fetuin.

the same commercially available glycoproteins did not verify this. The hydrolytic release of sialic acids reached final levels within 2 h at 80°C, irrespective of the glycoprotein hydrolysed (Fig. 4; *cf.*, ref. 33). The two sialic acid peaks, corresponding to Neu5Ac and Neu5Gc, are readily identified, and are well separated from the elution front. When the glycoprotein contents of these two sialic acids were determined with

TABLE I

SIALIC ACID COMPOSITION OF VARIOUS SAMPLES

Comparison of the present method with values given in the literature.

Sample	*Neu5Ac (%, w/w)*	*Neu5Gc (%, w/w)*	*Total amounts determined spectrophotometrically*	*Ref.*
Bovine				
submaxillary	13.0	7.9	20.9	
mucin type I-S	(11.6–13)[a]	(7.4)[a]	(22)[a]	12, 33
Human				
α_1-acid	10.6	ND[b]		
glycoprotein	(10.4)[a]	ND[b]	(10.8–14.7)[a]	12, 33, 37
Neu5Ac-α(2,3)-lactose	46.5	–	–	
	(47.2)[a]	–	–	31

[a] Values in parentheses were obtained from the literature.
[b] ND = not detected.

the present assay, the values obtained were essentially in agreement with those reported earlier (Table I).

The possibility that some O-acetyl groups may remain after this hydrolysis may also be considered. Such derivatives would then elute earlier than the corresponding sialic acid without the O-acetyl group. In fact, such extra peaks could be observed with considerably shorter times of hydrolysis. With the currently studied glycoproteins, however, no such peaks remained after the 2-h hydrolysis. The presence of detectable amounts of such O-acetylated derivatives from the studied preparations is also contradicted by the hydrolysis curve obtained (Fig. 4). In preparations where the possibility of remaining O-acetyl groups still must be considered, the problem may well be overcome by using a complementary alkaline hydrolysis.

It might seem advantageous to use neuramidase digestion as an alternative to acid hydrolysis, thereby avoiding problems with degradation. This is possible if the digestion is preceded by alkaline hydrolysis releasing O-acetate groups without affecting the sialic acids. Residues from the digestion buffer will, however, interfere with the per-O-benzoylation reaction. The use of neuramidase digests containing the normally recommended buffer would therefore necessitate a tedious purification of the released sialic acids or of the remaining glycoconjugate.

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