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# New sialic acids from biological sources identified by a comprehensive and sensitive approach: liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) of SIA quinoxalinones

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**Sialic acids are a family of 9-carbon carboxylated sugars, where different substitutions of the backbone define over 30 members. Biological roles of these substitutions have been missed until recently because of their low abundance and lability to conventional isolation/purification methods. This new approach characterizes sialic acids using electrospray ionization–mass spectrometry (ESI-MS) to monitor the HPLC separation of their DMB (1,2-diamino-4,5-methylenedioxy-benzene) derivatives (quinoxalinones). A combination of retention times and spectra characteristics allows definition of the type and position of the various substituents. This approach requires no previous purification, involving a simple derivatization reaction followed by direct injection on the microbore HPLC column. A complete spectrum, including molecular ions and CAD fragments of a sialic acid quinoxalinone, is obtained by injecting 10–20 pmol of the compound. Individual quinoxalinones can be purified by regular RP-HPLC and analyzed by direct-injection ESI-MS or LSIMS. Using this approach, we identified 28 different sialic acids, including the following new species: Neu5Gc9Lt (BSM), anhydro derivatives of Neu5Ac other than the 4,8-anhydro (horse serum hydrolyzates), KDN5(7)Ac and KDN5(7),9Ac$_2$ (amphibian *Pleurodeles waltl*), four isomers of Neu5Gc8MexAc and three anhydro derivatives of Neu5Gc8Me (glycolipids of the starfish *Pisaster brevispinus*), and Neu5Ac8S (in addition to Neu5Gc8S, in the glycolipids of the sea urchin *Lovenia cordiformis*). Results show the usefulness of LC-ESI-MS to study sialic acid diversity, and identification of small amounts of unexpected sialic acids or new members of their family.**

*Key words:* sialic acid/purification/electrospray ionization–mass spectrometry/quinoxalinone

## Introduction

Sialic acids are nine-carbon carboxylated sugars most frequently found in terminal position in glycoconjugates. The various substituents present on carbon 4, 5, 7, 8, and 9 generate a family of more than 30 different members. The substituent on carbon 5 can be either an amino, an acetamido, a glycolylamido, or a hydroxyl group and defines the four major types of sialic acids: neuraminic acid (Neu), N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and ketodeoxy-nonulosonic acid (KDN), respectively. Substituents of the hydroxyl groups present on carbons 4, 7, 8, and 9 can be either acetyl, lactyl, methyl, sulfate, or phosphate (Schauer, 1982; Varki, 1992). Sialic acids are involved in numerous biological properties of glycoconjugates, some of which are modulated by sialic acid modifications. For example, *O*-acetylation of sialic acids can alter the sensitivity to neuraminidases (Corfield *et al.*, 1986), and 9-*O*-acetylation masks the ligands of influenza A and B virus hemagglutinin (Rogers *et al.*, 1986), and of animal lectins such as CD22 (Sjoberg *et al.*, 1994) and sialoadhesin (Kelm *et al.*, 1994). These modifications have been shown to be tissue-specific (Klein *et al.*, 1994), and their expression is developmentally regulated (Varki, 1992). However, only a few examples of their significance have been published to date. This is due to (1) lack of sensitive, specific analytical techniques to investigate sialic acid substitutions; (2) their destruction during isolation and purification of the glycoconjugates to which they are attached due to their chemical lability; and (3) the presence of esterases in most biological materials (Varki, 1992). Furthermore, even if modified sialic acids survived isolation and purification, defining the position of each of the substituents in the backbone is not a straightforward matter.

The location of the different substituents on the sialic acid residue(s) could be studied by NMR spectroscopy while still attached to the particular glycoconjugate. However, this approach is limited by the need for pure molecules in nanomolar amounts. Any mass spectrometric approach (i.e., LSIMS, FAB-MS, ESI-MS, or MALDI-TOF) used for the study of intact, native, glycoconjugates yields intact molecular ions, and/or in addition to these, fragments resulting from the loss of sialic acids through cleavage of their glycosidic linkages. Retention of the charge on the sialic acid moiety produces fragments, corresponding to the total mass of each sialic acid residue including any substituents. In addition to dehydration products, no other fragments are obtained that could provide clues regarding the position of individual substituents. Furthermore, the sensitivity of this approach is limited because the glycoconjugate cannot be derivatized (i.e., permethylation, peracetylation, or reducing end tagging with 2-AB or BAP) if any probable substituent is to be preserved.

Study of sialic acid modifications has been attempted after release and purification by improving the methods to avoid any destruction (Varki and Diaz, 1984; Schauer, 1987). The characterization of released sialic acids can be achieved by chromatography: thin layer chromatography, gas–liquid chromatography (Schauer, 1987), or high performance liquid chromatography (Manzi *et al.*, 1990a). One of the techniques with higher sensitivity and resolving power is the reverse-phase

HPLC analysis of sialic acids as their fluorescent DMB (1,2 diamino-4,5-methylenedioxy-benzene) derivatives (Hara *et al.*, 1989; Manzi *et al.*, 1990a). These techniques have different degrees of complexity and sensitivity and, only when a mass spectrometer is used as detector in GLC, definitive proof of structure is obtained. However, serious limitations have been encountered when acetylated sialic acids are analyzed, possibly due to poor derivatization of some species and to the loss of acetyl groups during their elution from the columns at high temperature. The other approaches are limited to the comparison with available standards, and also by comigration of different sialic acids. Structural characterization of sialic acids can be done by NMR only after purification (Haverkamp *et al.*, 1982).

We have previously used FAB-MS to analyze mixtures of released and partially purified sialic acids, but the sensitivity of this method is limited (1–5 μg of native sample) unless sialic acids are peracylated (100 ng; Manzi *et al.*, 1990b). This analysis indicates the type and number of substituents, but defining the substitution positions requires the combination of the FAB-MS analysis with a time course of periodate oxidation using different conditions (Manzi *et al.*, 1990b). This increases the amount of sample required, and complicates the analysis.

We report here, the use of RP-HPLC-ESI-MS for the identification of sialic acid structures obtained in minimal amounts from biological sources. Although LSIMS (or FAB-MS) analysis of sialic acid quinoxalinones is feasible, the use of direct RP-microbore HPLC coupled to ESI-MS reduces the complexity of the approach and the CAD fragments obtained in this manner help identifying the positions of substitution. This approach involves a simple derivatization reaction (with DMB) that can be carried out without the need for purification of the sialic acids. Complete identification is achieved by a combination of ESI-MS patterns and the relative elution position as compared to those of known standards. The presence of base labile O-acyl esters can be confirmed by repeating the analysis after saponification.

The technique was first validated by analyzing the diverse group of sialic acids present in bovine submaxillary mucin (BSM), the polar lipid extract from the starfish *Pisaster brevispinus*, the glycoproteins of horse serum, and the jelly coat of the amphibian *Pleurodeles waltl*. These sources, already studied using other approaches (Schauer *et al.*, 1976; Reuter *et al.*, 1983; Manzi *et al.*, 1990b; Plancke *et al.*, 1994), contain most members of the sialic acid family reported up to date. Using this approach, we found new sialic acids, previously undetected in each one of these sources. Furthermore, we characterized the sialic acids from the sea urchin *Lovenia cordiformis*, demonstrating the capability of this approach to solve questions that could not be answered before.

## Results and discussion

### *LSIMS of sialic acids quinoxalinones*

DMB reacts with α-keto acids to produce 3-substituted 6,7 methylene dioxy-2(1H)-quinoxalinone fluorescent derivatives (Hara *et al.*, 1989). We will refer to these compounds with the name of the particular sialic acid followed with a capital Q to distinguish them from their parent molecules (i.e., Neu4,5AcQ = 3-(Neu4,5Ac$_2$)-6,7-methylenedioxy-2(1H) quinoxalinone). All specific positions (i.e., C4, C8, etc.) indicated correspond to the numbering within the neuraminyl substituent in position 3 of the quinoxalinone. For most naturally occurring sialic acids, these quinoxalinones can be separated by reverse-phase HPLC, and on-line fluorescence detection allows for the analysis of as little as 2.5 pmol of any of these compounds (Manzi *et al.*, 1990a). Substitution with acyl esters can be detected by saponification, before or after DMB derivatization, followed by rechromatography in the same conditions, and even 7-*O*-acylated species identified by mild base treatment that produces migration to the thermodynamically favored 9-position (Manzi *et al.*, 1990a). However, definitive proof of structure of each peak in an unknown sample would only be possible if each one of the pure individual sialic acids was available for coinjection. Furthermore, peak overlap and/or the presence of fluorescent reagent peaks can cause a problem in some instances.

We explored the use of semipreparative RP-HPLC for the isolation of the individual quinoxalinones. Since isocratic elution does not produce good resolution of mixtures containing many different sialic acids, we used an acetonitrile–methanol–water gradient (see *Materials and methods*). When these conditions were used for the analysis of the 6,7 methylenedioxy-2(1H)-quinoxalinones of the sialic acids from BSM, about 20 different fluorescent peaks were detected in an analytical run (Figure 1A). About 500 nmol of the same mixture of quinoxalinones were analyzed using the same column (see *Materials and methods*), the eluent was hand-collected in fractions according to the absorbance reading (the higher amount of derivatives injected allows for use of a UV detector set at 373 nm), on ice, and protected from the light. Ten semipreparative runs were done, and peaks representing the same fraction from the different runs were pooled. The fractions were immediately dried and reconstituted in a small volume of water, and an aliquot was reinjected for purity control (see Figure 1B). The remaining material was dried again and stored at −20°C until analyzed.

The dried quinoxalinones were analyzed by liquid secondary-ion mass spectrometry (LSIMS) using glycerol-thioglycerol 1:1 as the matrix. Each derivative showed a pseudo-molecular ion, both in positive and negative ion modes, with less than 5% of a dehydration product in each case (Figure 1B). Fractions containing more than one component (as indicated by the rechromatography of individual pools), clearly showed the different molecular ion species, in a ratio comparable to that resulting from on-line fluorescence detection. However, it was estimated that 10 μg (32.4 nmol) of Neu5Ac needed to be derivatized in order to obtain S/N of 10 in the LSI-MS analysis. Sialic acid quinoxalinones that were stored dried at −20°C did not show any significant difference in their LSI mass spectra after a 3 month period.

Next, we tried the direct analysis of sialic acid quinoxalinones, without RP-HPLC purification. Since the latter step not only allows fractionation of the different species, but also achieves elimination of excess reagents and salts, we extracted the DMB reaction mixture with ether. When this approach was used for BSM sialic acids, six different sialic acids were detected by loading about 30 μg of material on the LSIMS target (not shown). For samples directly analyzed after extraction, all sodium adducts of the molecular ions as well as ions resulting from the loss of one molecule of water were observed. Attempts at improving sensitivity by acylating the free hydroxyl groups (i.e., perdeuteroacetylation, perpropionylation) indicated a variable degree of substitution for different quinoxali-

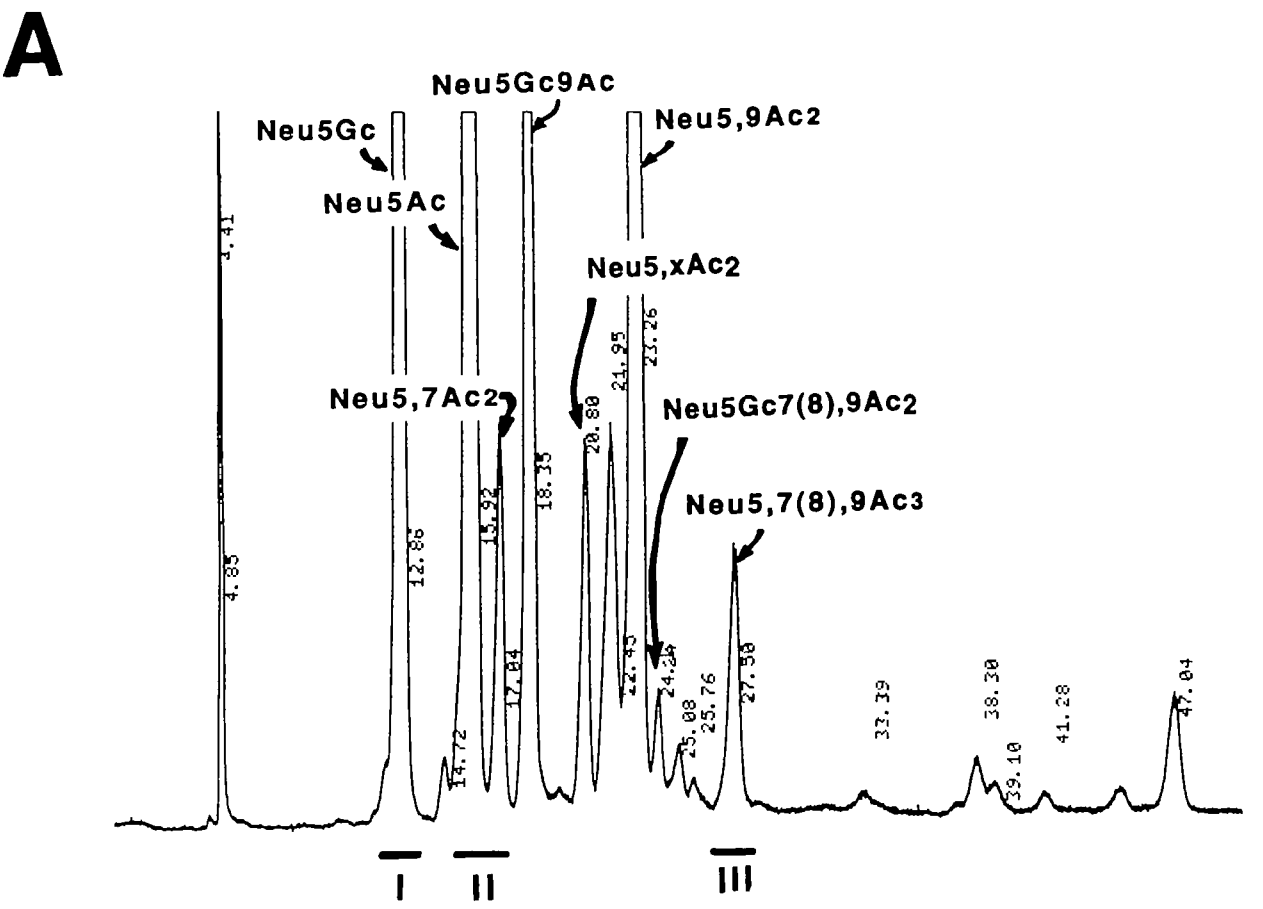


**Fig. 1.** RP-HPLC of sialic acid quinoxalinones. (A) RP-HPLC profile of DMB derivatized sialic acids from BSM monitored by on-line fluorescence detection; (B) see next page.

nones. Thus, the reaction was hard to reproduce and the LSI mass spectra were considerably more complicated.

*Electrospray ionization mass spectrometry of sialic acids quinoxalinones (SiaQ)*

Neu5Ac was derivatized with DMB and the reaction mixture directly injected into a microbore RP-HPLC column. The eluent was split (4:1) and monitored simultaneously by reading the absorbance at 373 nm, and by mass spectrometry through an electrospray ionization inlet (see *Materials and methods*). The ESI-MS spectra of the DMB derivative of Neu5Ac is characterized by two groups of ions (Figure 2). The first group corresponds to the molecular ion (m/z 426, $[M+H]^+$), and its sodium adduct (448, $[M+Na]^+$). The second group of ions results from collisional activation decompositions (CAD) generated in the electrospray transport region between the capillary and the first skimmer. Maximum information could be obtained from the fragmentation pattern when a potential of 100 V was used. This fragmentation produces the ion at m/z 408, $[M+H-18]^+$, corresponding to a first dehydration reaction, and ions A, B, C, and D at m/z 313, 295, 283, and 229. A possible interpretation of the CAD fragmentation of DMB derivatized Neu5Ac is given in Figure 3. The first dehydration occurs between the hydroxyl groups on carbons 4 and 8, since the production of a 6-member ring anhydro form is thermodynamically favored. This is supported by the fact that 4-substituted molecules fail to give this complex pattern (see below). Fragment A at m/z 313 could result from the elimination of acetamide and two molecules of water leading to the aromatization of the six-member ring. Fragments C and D could originate in the loss of formaldehyde and the fragmentation of the ring, respectively (see Figure 3), and fragment B could correspond to a further loss of water (see below). While these structures are speculative since fragmentation pathways need to be further studied by MS/MS and chemical derivatization approaches, they provide a plausible explanation to the fragments observed.

The molecular ion species of Neu5AcQ can be detected injecting as little as 5 pmol. However, typical CAD fragments are obtained, in the experimental conditions used (see *Materials and methods*), when at least 10–15 pmol are injected.

*Identification of sialic acids from bovine submaxillary mucin (BSM) by LC-ESI-MS*

The RP HPLC separation of DMB derivatized Sias from BSM is showed in Figure 4. The absorbance at 373 nm indicates the presence of at least 17 peaks (Figure 4A). Note the higher resolution achieved by the microbore column (compare Figures 1A and 4A). When the eluent from the microbore RP column is monitored by ESI-MS, identification of the type and number of substituents in each of these peaks can be simultaneously obtained. As indicated in Figure 4A, 14 of those peaks were identified as DMB derivatives of Sias.

The BSM sialic acid quinoxalinones were identified in the

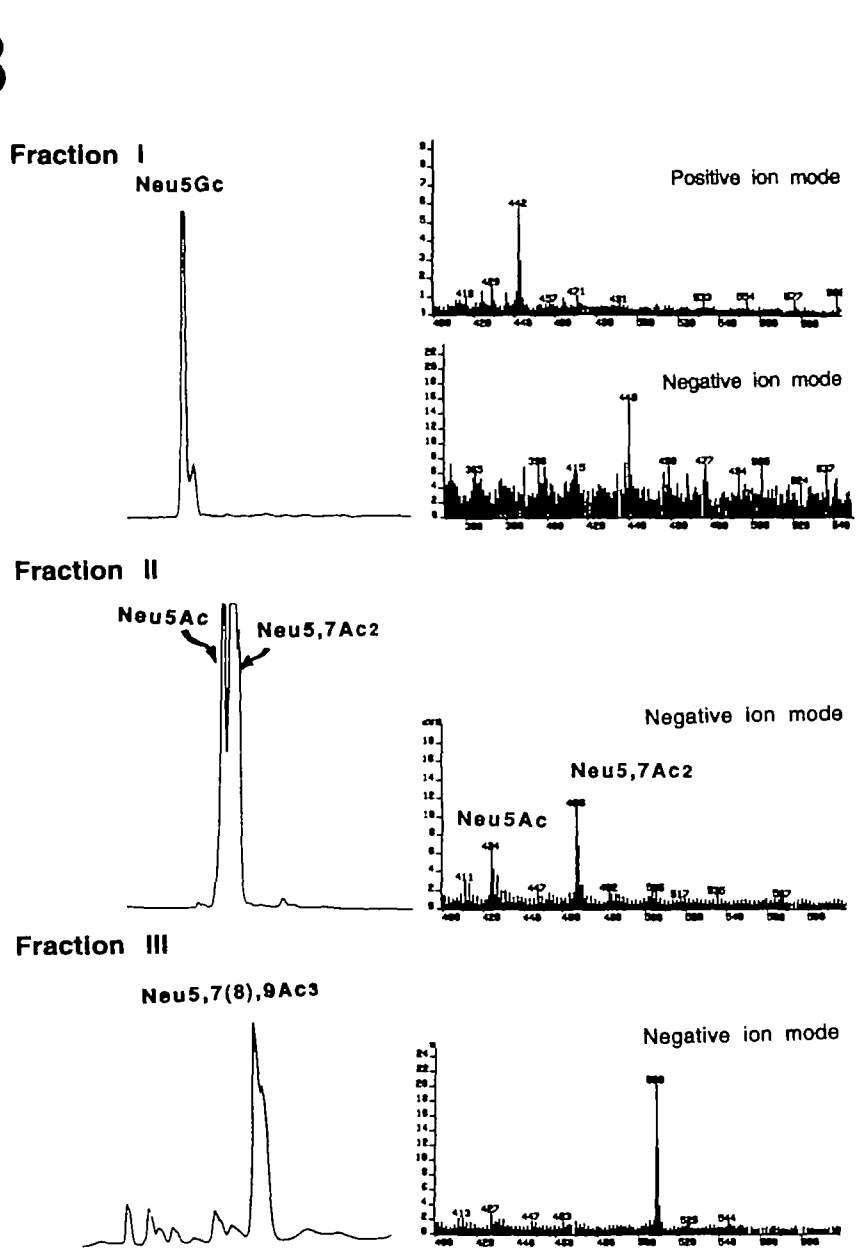


**Fig. 1.** (B) Rechromatography of fractions I, II, and III, and their LSIMS spectra.

following manner. Peak 1: Neu5GcQ, as indicated by the ions at m/z at 424, 442, 464 corresponding to $[M+H-18]^+$, $[M+H]^+$, $[M+Na]^+$, respectively. Fragmentation is similar to that of Neu5AcQ with the presence of ions A, B, C, and D. The $RRT_{Neu5Ac}$ (0.77) of this peak corresponds with the previously reported value (Manzi *et al.*, 1990a). Peak 2: mixture of two sialic acid quinoxalinones. The most abundant (2.1) is Neu5AcQ, and the minor component (2.2) yields ions at m/z 466, 484, and 506. This corresponds to the addition of 42 a.m.u to Neu5GcQ, indicating that the parent molecule was a mono-*O*-acetylated Neu5Gc. Because of the small proportion of this peak, CAD fragments were not obtained. However, a comparison of $RRT_{Neu5Ac}$ to those of the other mono-*O*-acetylated derivatives of Neu5Gc previously reported (Manzi *et al.*, 1990a) allowed to identified this peak as Neu5Gc7Ac. Peak 3: this peak ($RRT_{Neu5Ac}$ = 1.04) presents the same ions as compound 2.2. Thus, it is a second isomer of a mono-*O*-acetylated Neu5GcQ. Considering its $RRT_{Neu5Ac}$ (different than that of Neu4,5$Ac_2$, Neu5,7$Ac_2$, and Neu5,9$Ac_2$) and the CAD fragments (presence of m/z 313, 295, and 229, and absence of m/z 283 indicating that the O-acetyl group cannot be located on positions 4 or 7) we concluded this peak is Neu5Gc8Ac. Peak 4: Neu5,7$Ac_2$Q as indicated by the ions at m/z 450, 468, and 490, corresponding to $[M+H-18]^+$, $[M+H]^+$, $[M+Na]^+$, respectively. These ions indicate addition of an acetyl group (42 a.m.u) to Neu5AcQ. The comparison of $RRT_{Neu5Ac}$ with those of purified standards previously described for BSM sialics DMB derivatives (Manzi *et al.*, 1990a; Reuter and Schauer, 1994) indicates that peak 4 is Neu5,7$Ac_2$ ($RRT_{Neu5Ac}$ = 1.08). The fragmentation pattern of this mono-*O*-acetylated quinoxalinone is similar to that of the parent molecule with the presence of ions A, B, C, and D. In this case, ion A is formed by loss of acetamide, acetic acid and water. Peak 5: it is a minor component. The ESI-MS shows ions at m/z 496, 514, and 536

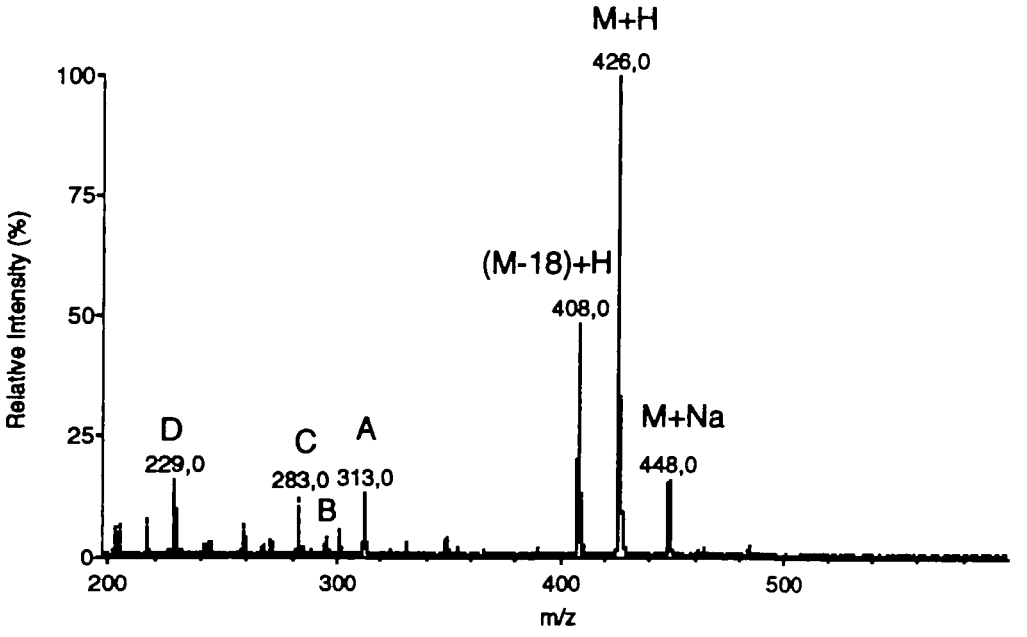


Fig. 2. ESI-MS mass spectrum of N-acetylneuraminic acid (Neu5Ac) after DMB derivatization (Neu5AcQ).

corresponding to the addition of 72 a.m.u. to Neu5Gc. This is in agreement with the presence of a lactyl ester. Although a lactyl ester of Neu5Ac (Neu5Ac9Lt) was previously reported (Schauer *et al.*, 1976), the existence of a similar derivative of Neu5Gc was never documented before. Peak 6: this is a third mono-*O*-acetylated isomer of Neu5GcQ, and its $RRT_{Neu5Ac}$ corresponds to that previously reported for Neu5Gc9AcQ (Manzi *et al.*, 1990a). Peak 7: it is a mono-*O*-acetylated isomer of Neu5AcQ as indicated by the ions at m/z 450, 468, and 490, corresponding to $[M+H-18]^+$, $[M+H]^+$, and $[M+Na]^+$, respec-

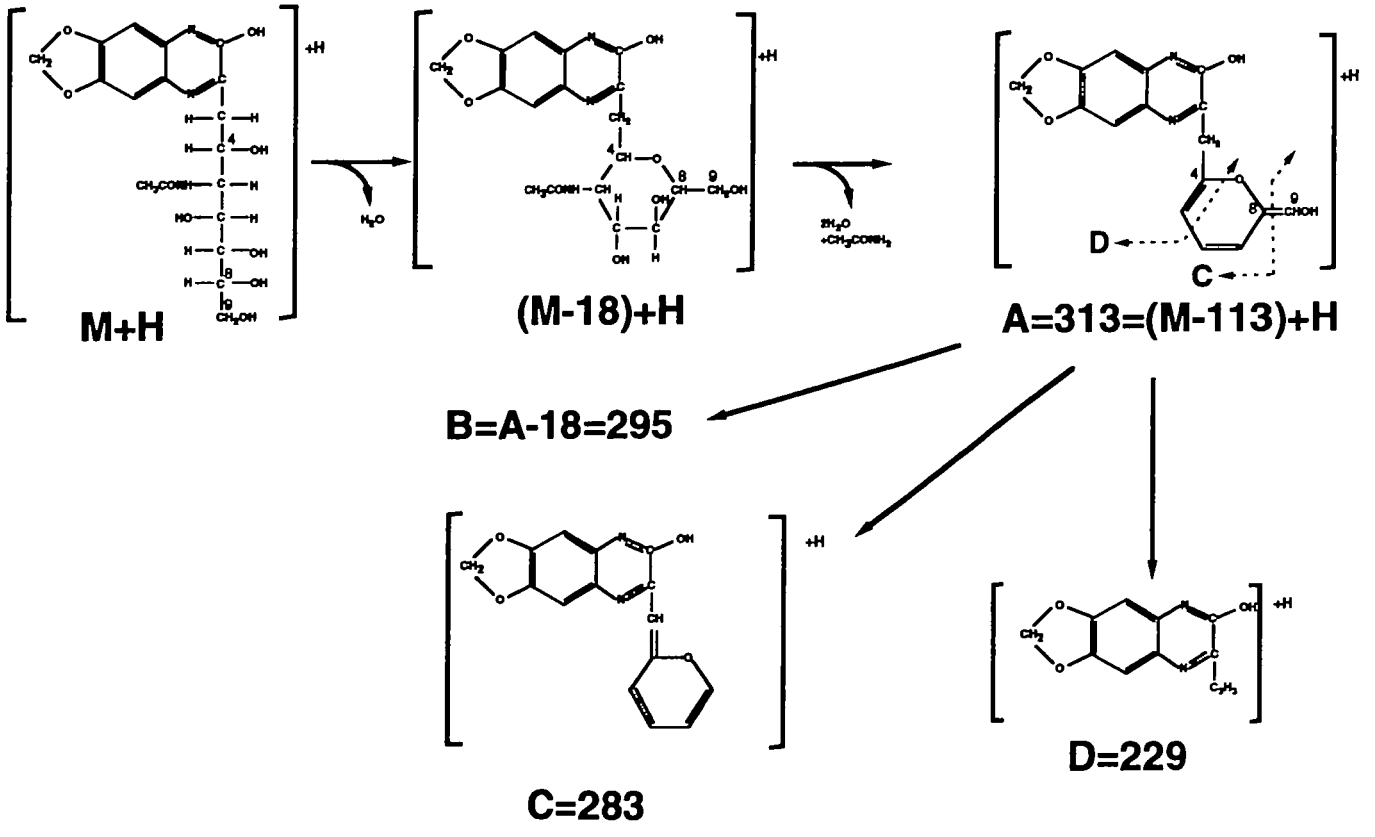


Fig. 3. Proposed fragmentation pathway for the characteristic ions observed in the ESI-MS of Neu5AcQ (structures are speculative and represent a possible explanation to the fragments observed).

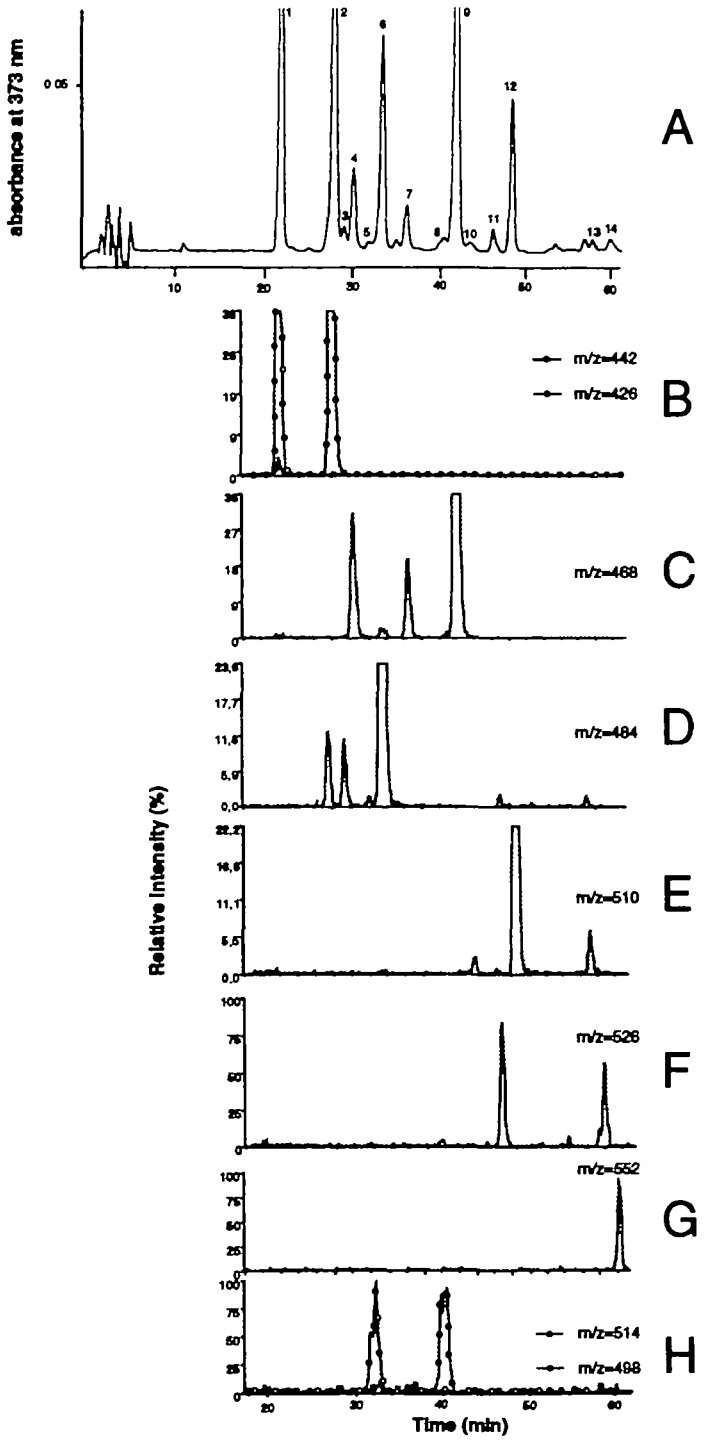


**Fig. 4.** LC-ESI-MS of sialic acid quinoxalinones. Elution profiles of DMB derivatized sialic acids from BSM on an ultrasphere ODS microbore HPLC column: (**A**) detected by measurement of the absorbance of the eluate at 373 nm; (**B–H**), Reconstruction of the molecular ion chromatograms of: Neu5Ac and Neu5Gc (**B**), mono-*O*-acetylated derivatives of Neu5Ac (**C**), mono-*O*-acetylated derivatives of Neu5Gc (**D**), di-*O*-acetylated derivatives of Neu5Ac (**E**), di-*O*-acetylated derivatives of Neu5Gc (**F**), tri-*O*-acetylated derivatives of Neu5Ac (**G**), and lactoylated derivatives of Neu5Ac and Neu5Gc (**H**).

tively. Peak 8: this is a minor component with molecular ions at m/z 480 $[M+H-18]^+$, 498 $[M+H]^+$, and 520 $[M+Na]^+$ corresponding to the addition of a lactyl group (72 a.m.u) to Neu5AcQ. The effect of the lactyl ester for both Neu5AcQ and Neu5GcQ molecules is similar to that of a 9-*O*-acetyl ester, being the lactyl esters of each sialic acid eluted slightly ahead of their 9-*O*-acetylesters. Peak 9: this is a third isomer of the mono-*O*-acetylated Neu5AcQ , and the $RRT_{Neu5Ac}$ corresponds to that previously reported for Neu5,9$Ac_2$Q (Manzi *et al.*, 1990a). Peak 10: The group of molecular ions at m/z = 492 $[M+H-18]^+$, 510 $[M+H]^+$, and 532 $[M+Na]^+$ correspond to the addition of 2 x 42 a.m.u to Neu5AcQ. Peak 11: in this case, the molecular ions at m/z = 508 $[M+H-18]^+$, 526 $[M+H]^+$, and 548 $[M+Na]^+$ correspond to the addition of 2 × 42 a.m.u to Neu5GcQ. Peak 12: it is an isomer of Neu5(7, 8, or 9)$Ac_3$, the $RRT_{Neu5Ac}$ (1.74) coincides with that reported for Neu5,7(8),9$Ac_3$ (Manzi *et al.*, 1990a). Peak 13: molecular ions at m/z 508, 526, and 548 indicate this is one of the possible isomers of Neu5GcA$c_2$Q. Peak 14: the ESI-MS contains ions at m/z 534 $[M+H-18]^+$, 552 $[M+H]^+$, and 574 $[M+Na]^+$, and the $RRT_{Neu5Ac}$ (2.16) corresponds to Neu5, 7, 8, 9 $Ac_4$ (Manzi *et al.*, 1990a).

As shown in Figure 4B–H, a rather simple dada manipulation strategy, the reconstruction of the individual chromatograms of characteristic ions, allows to rapidly decide if several positional isomers (same m/z) are present in a complex biological mixture of sialic acid quinoxalinones. The selected ion chromatogram reconstruction at m/z 468 indicates the presence of three mono-*O*-acetylated *N*-acetylneuraminic acid isomers with $RRT_{Neu5Ac}$ of 1.08, 1.31, and 1.51, respectively (see Figure 4C). When m/z 484 (indicating a mono-*O*-acetylated Neu5Gc) is reconstructed, three individual components with $RRT_{Neu5Ac}$ of 0.99, 1.04, and 1.19, respectively (see Figure 4D) were detected. Reconstruction of the chromatogram by extraction of the ion at m/z 510 indicates the presence of two isomers of di-*O*-acetylated Neu5Ac with $RRT_{Neu5Ac}$ of 1.57 and 1.74, respectively (see Figure 4E). Similarly, when m/z 526 is reconstructed (see Figure 4F), two different isomers of di-*O*-acetylated Neu5Gc ($RRT_{Neu5Ac}$ of 1.66 and 2.10) were detected.

The fragmentation resulting from the CAD of the sialic acid quinoxalinones gives information about the location of substituents. Fragments A and D at m/z 313 and 229 are present in all sialic acid derivatives, the ion at m/z 295 (B) is relatively more important as compared to fragments A and D when the sialic acid is substituted in 9. Fragment C at m/z 283 is present only if the sialic acid is not substituted in 8 or 9. A fragmentation pathway is proposed in Figure 5 for 9-*O*-acetylated sialic acid quinoxalinones. The rearrangement of the ion at m/z 313 giving a cyclohexa-diene-diol (m/z 295) is the only alternative for removing another water molecule consistent with the absence of the ion at m/z 283.

Therefore, even without knowing the relative retention times of standard quinoxalinones, it is possible to decide that peak 4 cannot be substituted in positions 4 (should not give $[M+H-18]^+$), 8, or 9 (should not produce m/z 283). In other cases, such as peaks 7 and 9, an ambiguity remains and it is not possible from the ESI-MS data alone to decide which peak corresponds to the derivative of 8 or 9 *O*-acetylated Neu5Ac. The comparison of relative retention times with those of known standards, available or reported in the literature (Manzi *et al.*, 1990a), answers this question. Furthermore, the fact that each one of these peaks represents an acyl ester of Sia can be confirmed by repeating the analysis after saponification.

In summary, 15 different sialic acids were identified in BSM by DMB derivatization-RP-HPLC-ESI-MS. One of these molecules, Neu5Gc9Lt was never detected before (see Figure 4H).

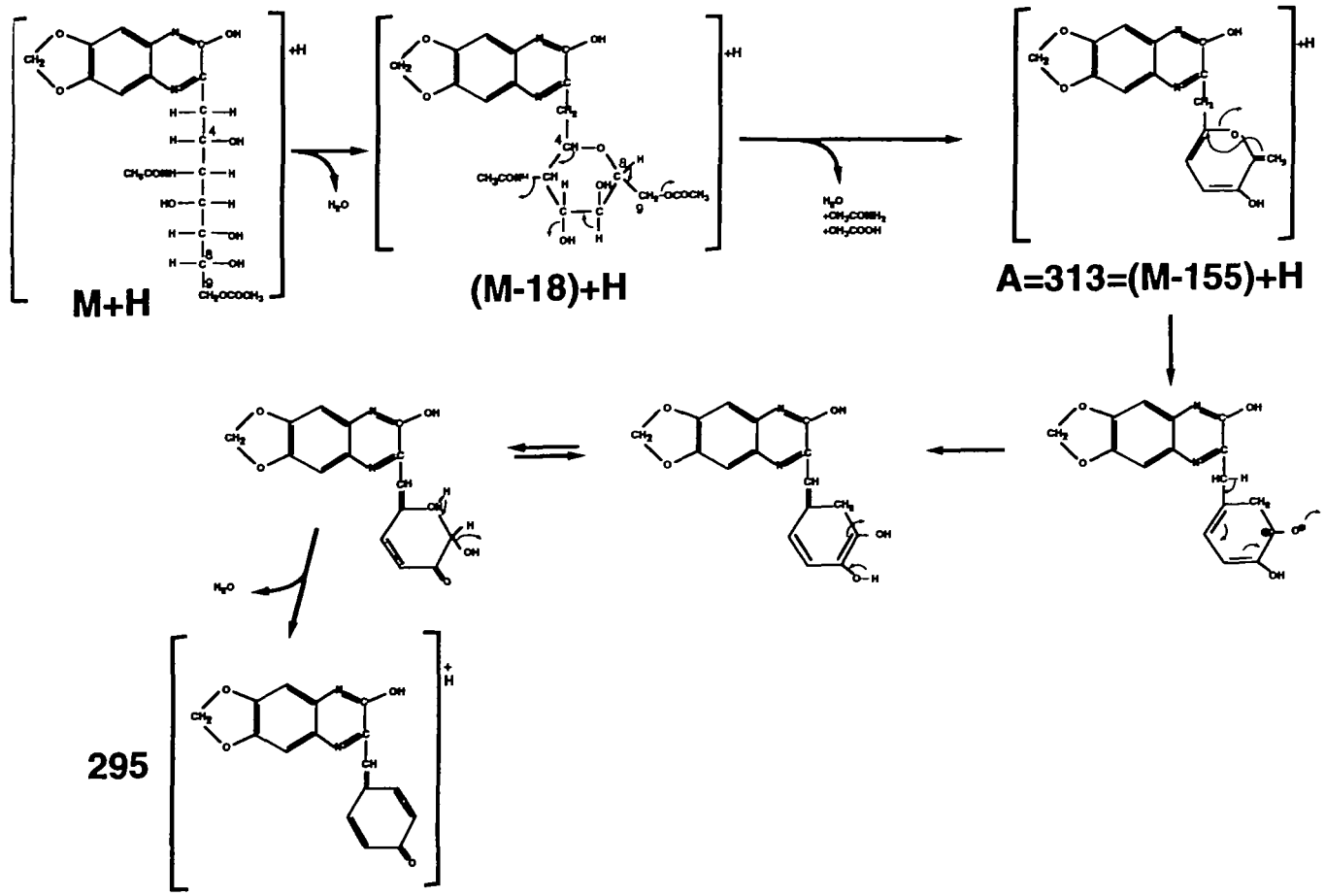


**Fig. 5.** Proposed fragmentation pathway for the characteristic ions observed in the ESI-MS of Neu5,9$Ac_2$Q (structures are speculative and represent a possible explanation to the fragments observed).

These sialic acids, their retention times and characteristic ions are summarized in Table I, and typical ESI-MS spectra are shown in Figure 6.

*The ESI-MS of 4-O-acetylated sialic acid quinoxalinones confirms the assignment of CAD fragments*

The ESI mass spectra of sialic acid quinoxalinones should be completely changed by the presence of a substituent in carbon 4 (see Figures 3 and 5). Therefore, we examined using this approach the sialic acids isolated from horse serum, a rich source of 4-*O*-acetylated species. The chromatogram shows in this case four major species with $RRT_{Neu5Ac}$ 0.77, 1.00, 1.39, and 1.60 (not shown). The spectra of the first two peaks are consistent with Neu5AcQ and Neu5GcQ. The $RRT_{Neu5Ac}$ of the third major component corresponds to that previously reported for 4,8 anhydro-N-acetyl-neuraminic acid quinoxalinone (Manzi *et al.*, 1990b). The ESI-MS shows ions at m/z 408, 430, and 446, corresponding to $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, respectively. Neu4,8an5Ac was previously found in acid hydrolyzates of collocalia mucoid (Saito *et al.*, 1989), between the products of the chemical treatment of Neu5Ac (Pozsgay *et al.*, 1987), and in the acid hydrolyzate of horse serum glycoproteins (Manzi *et al.*, 1990b). We have shown that the compound is formed from 4-substituted sialic acids through elimination of the 4-*O*-acetyl group under both, acidic and alkaline, conditions (Manzi *et al.*, 1990b).

The $RRT_{Neu5Ac}$ of the fourth sialic acid quinoxalinone indicates that this peak is the Neu4,5$Ac_2$ derivative (see Figure 6). The ESI-MS spectra showed the pseudo molecular ion $[M+H]^+$ at m/z 468, and its sodium adduct $[M+Na]^+$ at m/z 490. The absence of the ion $[M+H-18]^+$ indicates the inability of this molecule to directly undergo cyclization between carbons 4 and 8, thus confirming the proposed fragmentation pathway (Figure 3). Furthermore, the major ion observed corresponds to the loss of 60 a.m.u. (an acetic acid molecule), indicating the need to have the hydroxyl on C4 free to obtain the first cyclization.

A minor amount of Neu4Ac5GcQ ($RRT_{Neu5Ac}$ = 1.18, m/z 484 $[M+H]^+$, 506 $[M+Na]^+$, and 424 $[M+H-60]^+$), Neu4, 8an5GcQ ($RRT_{Neu5Ac}$ = 1.21, not resolved from the previous peak), and two other anhydro forms of Neu5Ac ($RRT_{Neu5Ac}$ of 1.32 and 1.49) were also detected. The last two anhydro forms correspond most likely to the less favored 5- and 7-member rings. Thus, this approach has allowed the detection of sialic acids present in a horse serum hydrolyzate that were not found before because of the lack of sensitivity.

*LC-ESI-MS of 2-keto-deoxy-nonulosonic acids (KDNs) quinoxalinones*

The jelly coat of *Pleurodeles waltl,* and other amphibians, have been shown to contain 2-keto-deoxy-nonulosonic acids (KDNs; Plancke *et al.*, 1994). In order to validate the LC-ESI-MS approach for these compounds, sialic acids were isolated

**Table I.** Relative retention times and characteristic ions of sialic acid quinoxalinones analyzed by microbore reverse-phase HPLC with on-line electrospray ionization mass spectrometry detection (LC-ESI-MS)

| Sialic acid | Abbreviation | Source | $R_{Neu5Ac}$ | M + H | CAD | |
|---|---|---|---|---|---|---|
| | | | | | (M-18) + H | Characteristic ions |
| *N*-Acetylneuraminic acid | Neu5Ac | Commercial | 1.00 | 426 | 408 | 313, 295, 283, 229 |
| *N*-Glycolylneuraminic acid | Neu5Gc | BSM | 0.78 | 442 | 424 | 313, 295, 283, 229 |
| Keto-deoxy-nonulosonic acid | KDN | Amphibian | 0.65 | 385 | 367 | 313, 295, 283, 229, 205 |
| 4-*O*-Acetyl-*N*-acetylneuraminic acid | $Neu4,5Ac_2$ | Horse serum | 1.60 | 468 | A | 408, 313, 283, 229 |
| 7-*O*-Acetyl-*N*-acetylneuraminic acid | $Neu5,7Ac_2$ | BSM | 1.08 | 468 | 450 | 313, 295, 283, 229 |
| 8-*O*-Acetyl-*N*-acetylneuraminic acid | $Neu5,8Ac_2$ | BSM | 1.31 | 468 | 450 | 313, 295, 229 |
| 9-*O*-Acetyl-*N*-acetylneuraminic acid | $Neu5,9Ac_2$ | BSM | 1.51 | 468 | 450 | 313, 295, 229 |
| 7,9-Di-*O*-acetyl-*N*-acetylneuraminicacid | $*Neu5,7,9Ac_3$ | BSM | 1.74 | 510 | 492 | 313, 295, 229 |
| 8,9-Di-*O*-acetyl-*N*-acetylneuraminic acid | $*Neu5,8,9Ac_3$ | BSM | 1.57 | 510 | 492 | ND |
| 7,8,9-Tri-*O*-acetyl-*N*-acetylneuraminic acid | $Neu5,7,8,9Ac_4$ | BSM | 2.16 | 552 | 534 | 313, 295, 229 |
| 4-*O*-Acetyl-*N*-glycolylneuraminic acid | Neu4Ac5Gc | Horse serum | 1.18 | 484 | A | 424, 313, 283, 229 |
| 7-*O*-Acetyl-*N*-glycolylneuraminic acid | Neu5Gc7Ac | BSM | 0.99 | 484 | 466 | ND |
| 8-*O*-Acetyl-*N*-glycolylneuraminic acid | Neu5Gc8Ac | BSM | 1.04 | 484 | 466 | 313, 295, 229 |
| 9-*O*-Acetyl-*N*-glycolylneuraminic acid | Neu5Gc9Ac | BSM | 1.19 | 484 | 466 | 313, 295, 229 |
| 7,9-Di-*O*-acetyl-*N*-glycolylneuraminic acid | $*Neu5Gc7,9Ac_2$ | BSM | 1.66 | 526 | 508 | 313, 295, 229 |
| 8,9-Di-*O*-acetyl-*N*-glycolylneuraminic acid | $*Neu5Gc8,9Ac_2$ | BSM | 2.10 | 526 | 508 | 313, 295, 229 |
| 5(7)-*O*-Acetyl-keto-deoxy-nonulosonic acid | KDN5(7)Ac | Amphibian | 0.94 | 427 | 409 | 313, 283, 245, 217, 205 |
| 9-*O*-Acetyl-keto-deoxy-nonulosonic acid | KDN9Ac | Amphibian | 1.07 | 427 | 409 | 391, 313, 295, 245, 229, 217, 205 |
| 5(7),9-Di-*O*-acetyl-keto-deoxy-nonulosonic acid | $KDN5(7),9Ac_2$ | Amphibian | 1.44 | 469 | 451 | 313, 295 |
| 9-*O*-Lactyl-*N*-acetylneuraminic acid | Neu5Ac9Lt | BSM | 1.45 | 498 | 480 | 313, 295, 229 |
| 9-*O*-Lactyl-*N*-glycolylneuraminic acid | Neu5Gc9Lt | BSM | 1.14 | 514 | 496 | ND |
| 8-*O*-Methyl-*N*-glycolyneuraminic acid | Neu5Gc8Me | Starfish | 0.98 | 456 | 438 | 313, 295, 229 |
| 8-*O*-Methyl-7-*O*-acetyl-*N*-glycolylneuraminic acid | Neu5Gc7Ac8Me | Starfish | 1.41 | 498 | 480 | 430, 316, 313, 229 |
| 8-*O*-Methyl-9-*O*-acetyl-*N*-glycolylneuraminic acid | Neu5Gc8Me9Ac | Starfish | 1.90 | 498 | 480 | 316, 313, 295, 229 |
| 8-*O*-Sulfo-*N*-glycolylneuraminic acid | Neu5Gc8S | Sea urchin | 0.16 | 522 | A | 464, 442, 424, 313, 229 |
| 8-*O*-Sulfo-*N*-acetylneuraminic acid | Neu5Ac8S | Sea urchin | 0.17 | 506 | A | 426 |
| 4-8-Anhydro-*N*-glycolylneuraminic acid | Neu4,8an5Gc | Horse serum | 1.21 | 424 | A | 313, 283, 229 |
| 4-8-Anhydro-*N*-acetylneuraminic acid | Neu4,8an5Ac | Horse serum | 1.39 | 408 | A | 313, 283, 229 |

*Assignments may be interchanged. A, absent. ND, not determined.

using acid hydrolysis followed by dialysis (see *Materials and methods*), and derivatized with DMB.

In this case, the first peak eluted from the RP HPLC column ($RRT_{Neu5Ac}$ = 0.61) corresponds to keto-deoxy-nonulosonic acid (KDN). This is indicated by the molecular masses observed in the ESI spectrum at m/z 367 $[M+H-18]^+$, 385 $[M+H]^+$ and 407 $[M+Na]^+$. Typical A, B, C, and D CAD fragments are also present (Figure 6). The other peaks correspond to acetyl esters of KDN. The most abundant of these *O*-acetyl esters ($RRT_{Neu5Ac}$ = 1.07) has been identified before as KDN9Ac by NMR (Plancke *et al.*, 1994). The ESI-MS (molecular species at m/z 409, 427, and 449, and CAD fragments A, C, and D) is consistent with KDN9AcQ. The rest of the *O*-acetylated species were never before detected.

Two minor acetyl esters were eluted at $RRT_{Neu5Ac}$ of 0.94 and 1.44, respectively. The first derivative is a mono-*O*-acetylated KDN with the same molecular ion than KDN9AcQ. The CAD fragments include m/z 205, 217, 245, 271, 283, and 295. The latter, together with the presence of a $[M+H-18]^+$ ion at m/z 409, indicates that the *O*-acetyl group cannot be located on positions 4, 8, or 9. Thus, this compound could be acetylated either in position 5 or 7. The absence of a CAD fragment at m/z 229, and the lack of loss of acetic acid, indicate that most likely this compound is acetylated in position 5 (KDN5AcQ). Thus, the fragment at m/z 245 should originate from the consecutive loss of acetate (small proportion of m/z 367 is observed) followed by the loss of three molecules of water and ring cleavage. The second derivative is a di-O-acetylated KDN showing molecular ions at m/z 451 $[M+H-18]^+$, 469 $[M+H]^+$, and 491 $[M+Na]^+$. The presence of an acetyl group in carbon 4 can be ruled out because of the formation of a dehydration product. Furthermore, the presence of CAD ions at m/z 313 and 295, and the absence of m/z 229, indicate this molecule is most likely $KDN5,9Ac_2Q$. Definitive proof of structure of these molecules by NMR spectroscopy is in progress in our laboratory.

### *LC-ESI-MS of quinoxalinones of sialic acids containing O-methylethers*

The starfish *Pisaster brevispinus* was previously shown to contain Neu5Gc, Neu5Gc8Me9Ac, and $Neu5Gc7,9Ac_28Me$ (Manzi *et al.*, 1990b). When the mixture of partially purified sialic acids, obtained by acid treatment of the total polar lipids, was derivatized with DMB and submitted to LC-ESI-MS, 10 different sialic acid derivatives were found. The most abundant species (66.2%) is Neu5Gc8Me ($RRT_{Neu5Ac}$ = 0.98). In addition to the typical molecular ions (m/z 438, $[M+H-18]^+$; m/z 456, $[M+H]^+$; and m/z 478, $[M+Na]^+$), the ESI-MS of Neu5Gc8Me contains fragment D, as well as those arising from the consecutive loss of two water molecules and acetamide, and the loss of those plus another water molecule. The above mentioned fragments have a different structure than those depicted in Figure 5, since C8 is substituted in this case. Fragment C is absent due to the same reason (Figure 6).

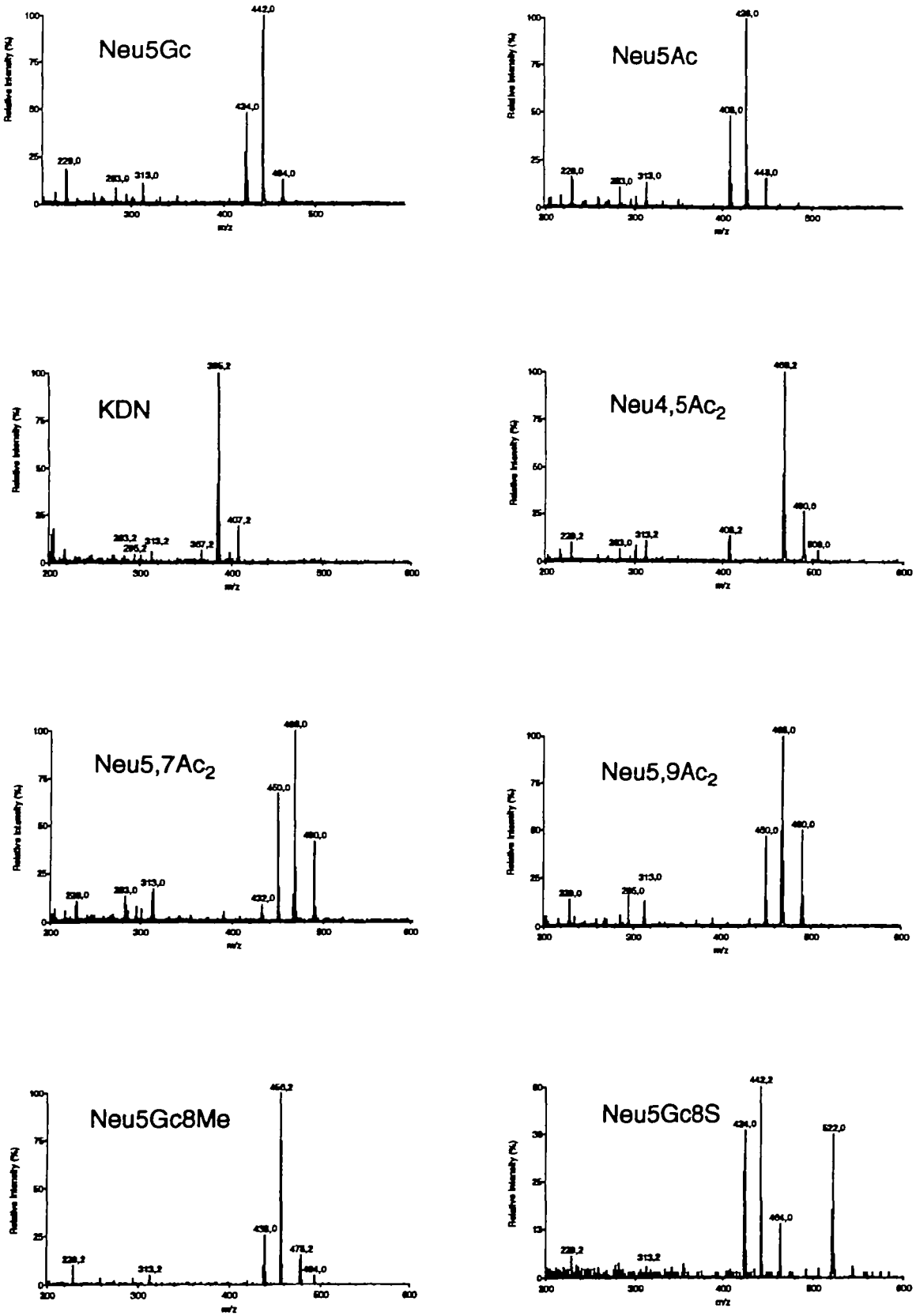


Fig. 6. ESI-MS mass spectra of typical sialic acid quinoxalinones. The source of the sialic acid is indicated between parentheses: Neu5Gc (BSM), Neu5Ac (commercial), KDN (*Pleurodeles waltl*, Neu4,5$Ac_2$ (horse serum), Neu5,7$Ac_2$ and Neu5,9$Ac_2$ (BSM), Neu5Gc8Me (*Pisaster brevispinus*), Neu5Gc8S (*Lovenia cordiformis*).

Four of the peaks detected correspond to Neu5Gc quinoxalinones containing one methyl group and one acetyl group. Three of these compounds ($RRT_{Neu5Ac}$ of 1.41, 1.86, and 2.05, respectively), are present in very minor proportions (<2%) while the fourth one ($RRT_{Neu5Ac}$ = 1.90), represents 6.1% of the total sialic acids in the mixture. All these peaks show the typical molecular ions and their sodium and potassium adducts (m/z 498, 520, and 536), and a first dehydration product (m/z 480). Among the CAD fragments, the ion at m/z 316 is a major ion for the first three quinoxalinones and indicates that etherification of the hydroxyl group at C8 blocks the cyclization between C4 and C8. This fragment might result from cyclization between the hydroxyl group of the glycolyl moiety and the hydroxyl group at C6, involving dehydration and an elimination of the side chain. The ESI mass spectrum of the first peak ($RRT_{Neu5Ac}$ = 1.41) contains an ion at m/z 430, corresponding to an intermediate step (before the side chain is eliminated) that is absent in the second peak ($RRT_{Neu5Ac}$ = 1.86). This indicates an stabilization of this ion that could be due to the presence of an acetyl group in C7. In the last quinoxalinone ($RRT_{Neu5Ac}$ = 2.05), the ion at m/z 316 is absent while prominent fragments are observed at m/z 214, 219, and 229. This points to the direct cleavage of the chain leaving short (C2 or C3) stubs attached to the aromatic ring. The most abundant isomer ($RRT_{Neu5Ac}$ = 1.90) should be acetylated at C-9 according to previous results (Manzi *et al.*, 1990b). Any further assignment of the position of substitution would be speculative, since all possible isomers would theoretically produce a similar combination of fragment ions. Furthermore, the possibility of a different location of the methyl group cannot be ruled out.

Also, the greater sensitivity of the ESI-MS approach, as compared to the previously used FAB-MS (Manzi *et al.*, 1990b) allowed us to detect the presence of one anhydro derivative of Neu5Gc ($RRT_{Neu5Ac}$ = 1.22) and three anhydro derivatives of Neu5Gc8Me ($RRT_{Neu5Ac}$ = 1.59, 1.69, and 1.75). The ESI mass spectra of Neu4,8an5GcQ shows the molecular ion at m/z 424, and its sodium adduct at m/z 446, and CAD fragments at m/z 313 (minor) and m/z 229. The ESI mass spectra of the anhydro forms of Neu5Gc8Me are characterized by two major ions at m/z 438 and 460, corresponding to the molecular ion and its sodium adduct, respectively. The first peak at $RRT_{Neu5Ac}$ = 1.59 (1.3%) shows a major CAD fragment at m/z 205. This ion is consistent with a 4,7-anhydro ring yielding the two fragments upon fragmentation of the C3–C4 bond. The other two anhydro forms represent 9.2% and 8.1%, respectively, of the total sialic acids in the mixture. Both peaks show CAD fragments at m/z 229, 259, 295, and 313. However, the second peak also has a fragment at m/z 268 that is not present in the other anhydro forms of Neu5Gc8Me (not shown). As before, definitive proof of structure will require purification of these molecules and NMR analysis.

Unfortunately, the previously described di-*O*-acetylated derivative of Neu5Gc8Me (Manzi *et al.*, 1990b) was not detected here. This may be because the retention time of this compound is longer than the final time employed for the run.

### *Use of the LC-ESI-MS approach for the analysis of unknowns: identification of sulfate esters of sialic acids*

Substitution of the hydroxyl group at the 8 position of sialic acids with a sulfate ester has so far been reported in the gangliosides of different species of sea urchin (Kochetkov *et al.*, 1976), and in the gangliosides of bovine gastric mucosa (Slomiany *et al.*, 1981a,b). Sea urchin gangliosides were shown to be completely resistant to the action of *Vibrio cholerae* sialidase (Kochetkov *et al.*, 1976). On the other hand, bovine gastric mucosal gangliosides were reported to contain Neu5Ac8S and Neu5Gc8S in mono- and disialosyl-galactosylceramides, and both sulfated gangliosides were shown to be completely resistant to the action of *Clostridium perfringens* sialidase (Slomiany *et al.*, 1981a,b).

The difficulty in the analysis of sulfated carbohydrates is a very well known fact. Sulfate esters can be cleaved in both acidic and alkaline conditions, and different strategies for determining the substitution position present different difficulties (Scudder *et al.*, 1986; Kubo *et al.*, 1990; Dell *et al.*, 1991). In all above mentioned cases, the identification of 8-*O*-sulfate esters in sialic acids had been done by direct analysis of the intact glycoconjugates containing these residues. This is feasible when enough material is available, and can be accomplished by NMR spectroscopy. However, for regular screening of biological samples a more sensitive approach needs to be used.

The presence of sulfate esters in the polar lipids from the sea urchin *Lovenia cordiformis* was indicated by HPTLC analysis of the total lipid extract. A considerable shift in Rf towards a faster migrating band was obtained after submitting the extract to desulfation conditions (not shown). We isolated total sialic acids from the polar lipids (see *Materials and methods*) and submitted this mixture to anion-exchange chromatography in the usual conditions employed for the purification of sialic acids (see *Materials and methods*). In this case, an anomalous behavior was observed: 64% of the sialic acids were not retained by the Dowex AG3x4A column, 19% were eluted with 1 M formic acid, and 17% required 5 M formic acid to be eluted. Although the three sialic acid groups were always obtained, the percentage of sialic acids not retained by the Dowex AG3X4A column varied considerably between experiments, indicating that these might be degradation products of the sulfated species. A sulfated derivative is expected to bind tightly to the anion exchange resin, requiring high concentration of formic acid for elution. The two fractions eluted from the anion exchange column were analyzed by negative ion FAB-MS, but no sensible data was obtained. Only masses expected for Neu5Gc were obtained after permethylation, or reduction/perdeuteroacetylation, and analysis by positive ion FAB-MS.

When total sialic acids were released with acid from the polar lipids of *Lovenia cordiformis,* derivatized with DMB and analyzed by ESI-MS, a clear identification of the sulfated species was possible. The RP HPLC profile showed two major peaks ($RRT_{Neu5Ac}$ of 0.16 and 0.77), and two minor peaks ($RRT_{Neu5Ac}$ of 0.96 and 1.00). The short retention time of the first derivative is indicative of a polarity higher than that of Neu5Ac and Neu5Gc. The ESI-MS of the first major component ($RRT_{Neu5Ac}$ = 0.16) contains a molecular ion at m/z 522 corresponding to the addition of a sulfate group to Neu5GcQ (see Figure 6). The rest of the spectrum is identical to that of Neu5GcQ. The other major sialic acid is Neu5Gc as indicated by the retention time and by the ESI-MS spectrum of its quinoxalinone. The two minor components can be identified as Neu5AcQ ($RRT_{Neu5Ac}$ = 1.00) and Neu5Gc8MeQ ($RRT_{Neu5Ac}$ = 0.96).

Reconstruction of the ion chromatograms corresponding to m/z 426 and 506, indicated the presence of another very minor component (not detected by absorbance at 373nm). The molecular ion of this product ($RRT_{Neu5Ac}$ = 0.17) is consistent

with the addition of one sulfate group to a Neu5Ac molecule. To the best of our knowledge, Neu5Ac8S was never before found in *Echinodermata*.

We have not found any other isomer of mono-*O*-sulfate-NeuGc or Neu5Ac. While Neu5Gc9S was recently found on the poly-Neu5Gc chains of sea urchin egg cell surface glycoprotein (Kitazume *et al.*, 1996), there is no report of 9-*O*-sulfation of Sias in glycolipids.

*Conclusions*

This article describes a new approach for the identification of sialic acids isolated in limited amounts from biological sources. The approach is based on the LC-ESI-MS analysis of sialic acids as their methylenedioxy-quinoxalinones, and can be used for any member of the sialic acids family, with the exception of those that do not contain an α-keto-acid group since they cannot form quinoxalinones. However, these sialic acids (i.e., 2-deoxy-2,3 didehydro-N-acetyl, Neu2en5Ac, or N-glycolyl-neuraminic acid, Neu2en5Gc) occur only in biological fluids in the free form and have never been described in glycoconjugates.

The strategy was validated by analyzing mixtures of sialic acids previously characterized by other methods (i.e., BSM, horse serum). Application of this approach allowed to identify minor proportions of naturally occurring sialic acids that were not detected before such as Neu5Gc9Lt in BSM, anhydro derivatives of Neu5Ac other than the 4,8-anhydro in horse serum hydrolyzates, KDN5(7)Ac and KDN5(7),9$Ac_2$ in the amphibian *Pleurodeles waltl*, four mono-*O*-acetylated isomers of Neu5Gc8Me and three anhydro derivatives of Neu5Gc8Me in the glycolipids of the starfish *Pisaster brevispinus*. Furthermore, the approach is appropriate for the characterization of sulfate esters of sialic acids not detected using other methodologies: both Neu5Ac8S, and the previously reported Neu5Gc8S were found in the glycolipids of the sea urchin *Lovenia cordiformis*. A total of 28 members of the sialic acid family, differing in the position and nature of their substituents, were successfully identified by interpreting the characteristic molecular and CAD ions of their ESI-MS in combination with their relative retention times, as compared to those of known standards (see Table I for a summary).

The major advantages of this technique are its simplicity and sensitivity. Sialic acids do not need to be purified before analysis, the approach involves only a one-step derivatization reaction, and the reaction mixture is directly injected in the microbore HPLC column (that achieves fractionation of complex mixtures), and monitored by ESI-MS that indicates the type of structure present. While 5 pmol of a sialic acid quinoxalinone can be detected (molecular ion species), typical CAD fragments that help in the identification of individual isomers are obtained in the experimental conditions used (see *Materials and methods*) when at least 10–15 pmol is injected. For a complex mixture, such as that from BSM (15 different sialic acids), all sialic acids can be characterized in a single run when at least 100 pmol is injected. It is necessary to point out that, in this case, there are four major components representing more than three quarters of the total sialic acids while other peaks are present in very minor proportions. Without MS monitoring (m/z data), sialic acids with very similar chromatographic behavior (i.e., 9-*O*-lactyl-Neu5Gc, RRTNeu5Ac = 1.14, and 9-*O*-acetylNeu5Gc, RRTNeu5Ac = 1.19) could not be distinguished leading to erroneous conclusions about their biological significance. Therefore, we have demonstrated that LC-ESI-MS is a valuable tool for the study of sialic acid diversity, and also for the characterization of new members of this family.

## Materials and methods

*Chemicals and solutions*

HPLC solvents were from Carlo Erba (Milano, Italy), or Fisher Scientific (USA). All other chemicals were of reagent grade or better, and were from commercial sources.

*Sources and purification of sialic acids*

N-acetyl neuraminic acid (NeuAc) was from Pfanstiehl (Waukegan, Illinois) or Kantoishi Pharmaceutical (MECT) Co., Tokyo, Japan; and N-glycolyl-neuraminic acid (Neu5Gc) was from Sigma. Sialic acids from bovine submaxillary mucin, equine serum, and the total lipid extract of the starfish *Pisaster brevispinus*, were obtained as previously described in Manzi *et al.*, 1994b.

*Sialic acids from the jelly coat of the amphibian* Pleurodeles waltl. The jelly coat material (7 mg), a generous gift of G. Strecker (University of Lille), was treated with 5 ml of 2 M acetic acid for 2.5 h at 80°C. The suspension was dialyzed against 20 volumes of water for 20 h with one change after 4 h (1000 MW cut-off tubing). The lyophilized diffusate was directly analyzed.

*Sialic acids from the sea urchin* Lovenia cordiformis. *Lovenia cordiformis* specimens were purchased from Pacific Biomarine (Los Angeles, CA). The gonads of 10 female sea urchins (232.8 g) were finely minced, washed with methanol, and total lipids extracted into 10 vol of chloroform/ methanol mixtures (2:1, 1:1, and 1:2, v/v), and finally into chloroform:methanol:water, 10:10:1 (v/v/v) using a Polytron homogenizer. The pooled extracts were taken to dryness in a rotary evaporator, dissolved in chloroform:methanol, 2:1 (v/v) and submitted to Folch partition (Folch *et al.*, 1957). The polar lipids from the upper layer of the Folch partition were recovered after dialysis against water, by lyophilization (yield = 88.5 mg). The sialic acids present in this total lipid extract (2 mg = 107 nmol sialic acid) did not show any *O*-acetylation as indicated by the lack of difference in the response of the TBA assay before and after saponification. A portion of the lipids (10.9 mg) were treated with 1.5 ml of 2 M acetic acid (in the presence of 1% BHT to inhibit lipid peroxidation) for 5 h at 80°C (this period was shown to cause maximum release). Only 72% recovery of sialic acids was obtained. The suspension was dialyzed for 24 h against 20 volumes of water (1000 MW cut-off tubing), and the diffusate containing the sialic acids was directly loaded on a Dowex AG 50 WX8 (H+) column (10 ml). The column was washed with water (100 ml) and the effluent lyophilized (yield = 420 nmol), dissolved in water, and loaded on a Dowex AG3X4A (HCOO-) column (1 ml). The column was first washed with 7 ml of 10 mM formic acid, and then eluted with 1 M and 5 M formic acid. Of the total sialic acids, 64% were not retained by the column, 19% were eluted with 1 M formic acid, and 17% required 5 M formic acid to be eluted. However, co-chromatographed radiolabeled Neu5Ac behaved as expected (>90% of the radioactivity was recovered in the 1 M fraction).

*Analysis of sialic acids*

The 2-thiobarbituric acid (TBA) assay was used to assay free sialic acids (Warren, 1959).

*De-O-acetylation of free sialic acids*

De-*O*-acetylation was carried out (prior to derivatization with DMB) with 0.1 M NaOH for 30 min at 37°C and quenched by neutralization with 0.2 M HCl.

*Derivatization with DMB*

Samples were heated at 50°C for 2.5 h in the dark in 7 mM DMB, 0.75 M β-mercaptoethanol, and 18 mM sodium hydrosulfite in 1.4 M acetic acid (Hara *et al.*, 1989).

*High performance liquid chromatography*

DMB derivatized sialic acids were separated by RP-HPLC using a TSK-ODS 120T column (250 × 4.6 mm i.d.; particle size, 5 mm, TosoHaas, Montgomery, PA), or a C-18 column (250 × 4.6 mm; particle size 5 mm, Rainin, Emeryville, CA). Elution was achieved using a linear gradient from 7/14/79 to 11/14/75 of acetonitrile/50% methanol/water over 40 min, followed by 10 min at that solvent ratio, at 0.9 ml/min flow. No critical difference in performance was observed between the two columns. On-line fluorescence detection was

achieved with an FD-300 dual monochromator fluorescence detector (with a 24 μl flow-cell), Spectro Vision, Inc. Emission wavelength was set at 448 nm, and excitation wavelength at 373 nm, with a response time of 0.5 sec. For semipreparative fractionations, the absorbance of the eluent at 373 nm was measured on-line with an SP-8440 UV-visible detector, Spectra Physics. Microbore RP-HPLC was done on an ultrasphere ODS column (25 × 0.2 cm; particle size 5 μm, Beckman, Fullerton, CA) eluted with a linear two-step gradient of 3.85/7.00/89.15 to 7.15/7.00/85.85 (v/v/v) acetonitrile/methanol/water in 40 min, and from that ratio to 11.00/7.00/8.00 in 10 min. A flow rate of 0.2 ml/min was controlled with an Applied Biosystem 140B HPLC apparatus. Detection was achieved by simultaneous measurement of the absorbance of the eluate at 373 nm and by ESI-MS.

*Liquid secondary ion mass spectrometry*

Sialic acid quinoxalinones (5–50 μg) were dissolved in 5% acetic acid or methanol (5 μl), and loaded onto the stainless steel LSIMS target covered with a glycerol-thioglycerol matrix. Spectra were obtained in a VG analytical 70-SE mass spectrometer (La Jolla Cancer Research Foundation) fitted with a cesium ion gun. Both, positive and negative ion modes were used. Mass range m/z 100–1000 was scanned and cesium iodide was used as standard for mass calibration. All data were acquired and processed in a VG 11–250J data system.

*Electrospray ionization–mass spectrometry*

The mass spectrometric data was acquired on an API-I simple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada) fitted with an articulated, pneumatically assisted nebulization probe and an atmospheric pressure ionization source. The ion spray voltage was operated at 5 kV. The tuning and calibration solution consisted of an equimolecular mixture of poly(propylene glycol) (PPG) 425, 1000, and 2000 in 50/50/0.1 H2O/methanol/formic acid (v/v/v), 1 mM $NH_4OAc$. Spectra were acquired by scanning from m/z = 80 to m/z = 800 in 20 s with a step size of 0.2 a.m.u and a dwell time of 1 ms. The spectra were recorded at an orifice voltage of +100 V. The solvent from the HPLC was split with a ratio of 1 to 4, 40 μl/min being introduced into the ES source.

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## Abbreviations

Sia, sialic acid; Neu5Ac, *N*-acetyl-neuraminic acid; Neu, neuraminic acid; *N*-glycolyl-neuraminic acid; BSM, bovine submaxillary mucin; BHT, beta-hydroxy toluene; FAB-MS: fast-atom bombardment mass spectrometry; LSIMS: liquid secondary ion mass spectrometry; ESI-MS: electrospray ionization mass spectrometry; CAD: collisional activation decomposition; MALDI-TOF MS, matrix assisted laser desorption ionization mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; RP, reverse-phase; TLC, thin-layer chromatography; KDN, keto-deoxy-nonulosonic acid; TBA, 2-thiobarbituric acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride; 2-AB, 2-amino-benzamide; BAP, biotinylated aminopyridine; S/N, signal to noise ratio.

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