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STRUCTURAL ANALYSIS OF UNDERIVATIZED SIALIC ACIDS BY COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Mass spectra of chemically ionized, positive ions of underivatized N,O-acylated sialic acids, 2-deoxy-2,3-didehydro-N-acetylneuraminic acid and sialyl-α(2-3)-lactose were obtained by combined high-performance liquid chromatography—mass spectrometry, using a direct liquid inlet system. The mass spectra of the different compounds for which fragmentation schemes are proposed enable the differentiation between sialic acids, although the localization of O-substituents is not possible. However, since the various sialic acids separated well on high-performance liquid chromatography, combined high-performance liquid chromatography—mass spectrometry allowed their unequivocal characterization.

INTRODUCTION

Structural analyses of sialic acids have so far been performed by combined gas—liquid chromatography—mass spectrometry (GLC—MS) [1] or by nuclear magnetic resonance spectroscopy [2]. The general application of these methods, however, suffers from the facts that (i) relatively large amounts of carefully purified sialic acids (50—500 μg) are required, (ii) derivatization is necessary for GLC—MS analysis of sialic acids, and (iii) extensive purification and derivatization steps may lead to intramolecular migration of O-acetyl

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groups or to loss of sialic acids [3, 4]. Recently, a high-performance liquid chromatographic (HPLC) method has been developed for the sensitive analysis of different, naturally occurring N,O-acylated sialic acids and for studies of their metabolic reactions [5, 6]. A major advantage of this method is that derivatization and, in most cases, extensive purification of sialic acids are not required.

In the present study it is shown that HPLC, combined with chemical-ionization mass spectrometry, represents a powerful tool for the characterization of different underivatized sialic acids. It may be useful for the rapid analysis of micro-quantities of sialic acid mixtures, isolated from biological material available only in small amounts, or originating from enzyme reactions metabolizing sialic acids [3].

MATERIALS AND METHODS

Materials

Sialic acids were isolated from bovine, equine and porcine submandibular gland glycoproteins as described in ref. 7. Sialyllactose (Neu5Ac-α(2-3)-lactose)* was prepared from bovine colostrum [8]. Neu5Ac2en was purchased from Boehringer.

High-performance liquid chromatography

HPLC of sialic acids and sialyllactose was performed on a stainless-steel column (40 × 4.6 mm) filled with Aminex A-29, using a HPLC pump (Milton Roy, Model ConstaMetric III). Sialic acids were eluted with ammonium formate (20 m*M*, pH 6) in water—acetonitrile (4:1, v/v) at a flow-rate of 0.5 ml/min and 15—20 bars. Eluted sialic acids were monitored at 200 nm. For further details of this method see ref. 5.

High-performance liquid chromatography—mass spectrometry

For the direct liquid inlet technique, a Hewlett-Packard mass spectrometer (Model 5985 B) equipped with an LC—MS interface was used. HPLC conditions were the same as above, except that 3% of the HPLC effluent was transferred to the mass spectrometer source. At the source temperature of 250°C the buffer components are volatile and act as chemical-ionization reagent gas. In some cases pure sialic acids dissolved in water (5—10 μg per 10 μl) were applied directly without column, on-line to the mass spectrometer.

RESULTS AND DISCUSSION

Since in the combined HPLC—MS system only volatile salts can be used, the previously described sodium sulphate solution [5] was replaced by a water—acetonitrile mixture containing ammonium formate (or acetate). This buffer

*Abbreviations: Neu5Ac = N-acetylneuraminic acid; Neu5Gc = N-glycolylneuraminic acid; $Neu5,9Ac_2$ = N-acetyl-9-O-acetylneuraminic acid; further abbreviations for mono-, di-, and tri-O-acetylated sialic acids are used correspondingly; Neu5Ac2en = 2-deoxy-2,3-dide-hydro-N-acetylneuraminic acid. The sialic acid nomenclature corresponds to a proposal made at the Vth International Symposium on Glycoconjugates, held at Kiel-Damp in 1979.

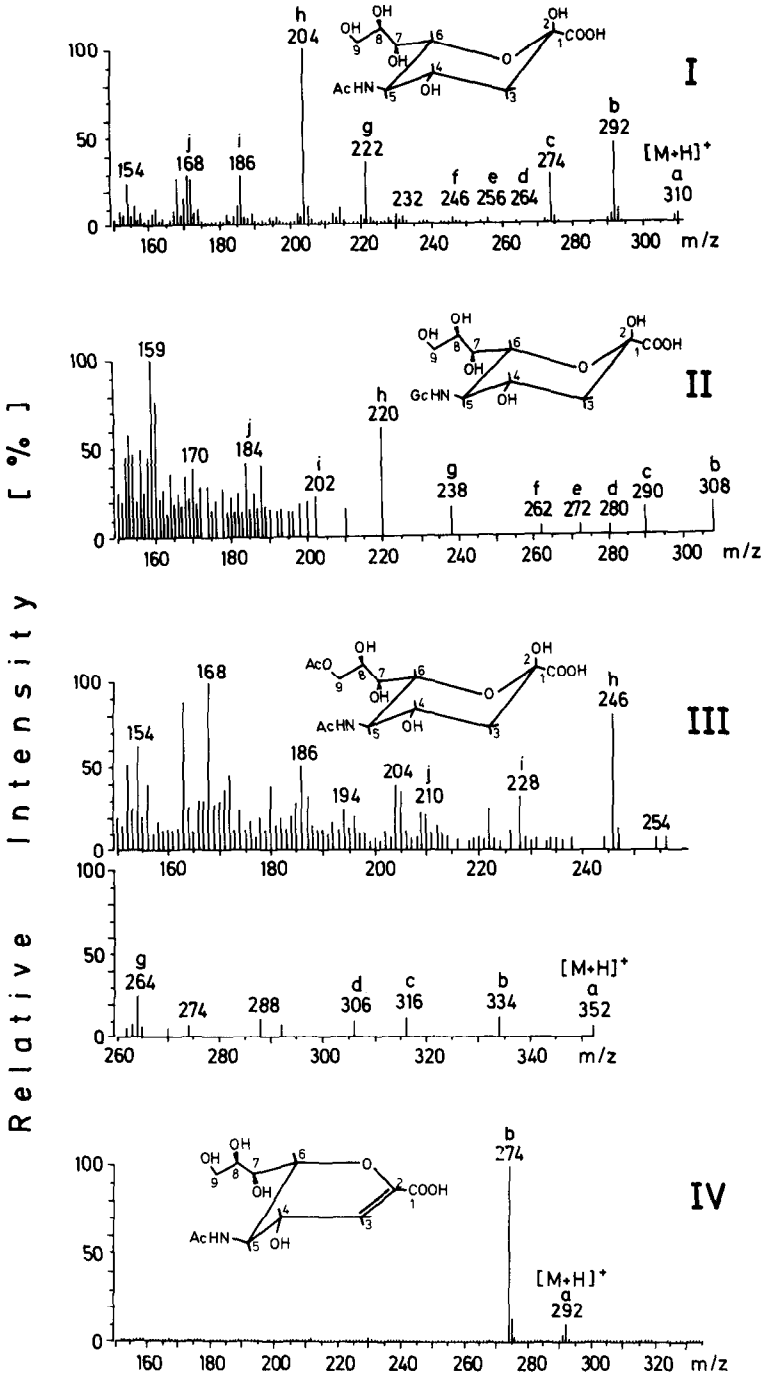

Fig. 1. Mass spectra of chemically ionized, positive ions of underivatized sialic acids obtained by combined HPLC—MS. I, Neu5Ac; II, Neu5Gc; III, Neu5,9Ac$_2$; and IV, Neu5Ac2en. The structural formula and [M + H]$^+$ fragments of sialic acids are shown. The [M + H]$^+$ fragment of Neu5Gc was not recorded in this spectrum. For other sialic acids and for the explanation of fragments see Figs. 2 and 3, and Table I.

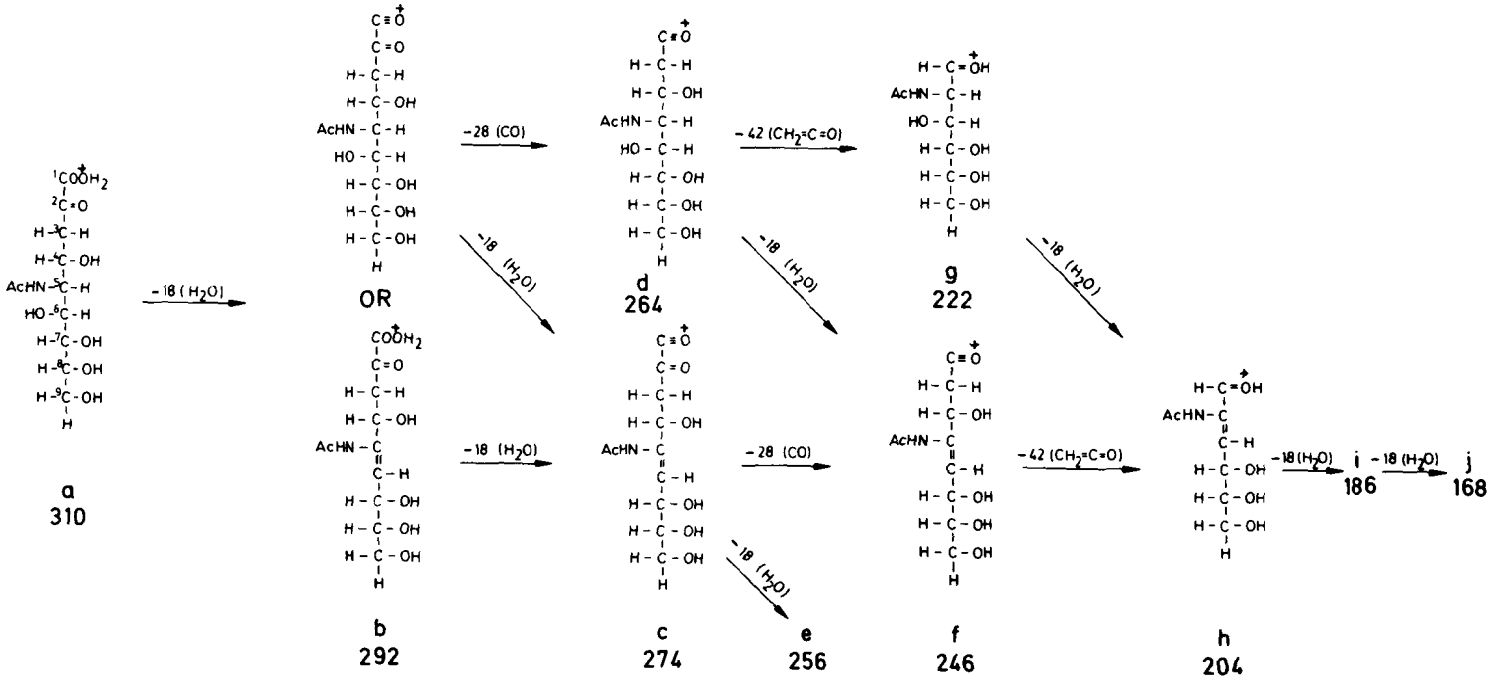

Fig. 2. Mass fragmentation pathway proposed for chemically ionized, positive ions of underivatized Neu5Ac (open-chain form) by combined HPLC—MS.

proved to be most suitable for the recording of mass spectra of underivatized sialic acids, as it volatilizes at the source of the mass spectrometer at a temperature of 250°C and thus acts as chemical-ionization gas. A disadvantage of these volatile salts, as compared to the sodium sulphate solution, however, is a reduction of the sensitivity of the photometric determination of sialic acids by a factor of 10—50.

The mass spectra of Neu5Ac, Neu5Gc, Neu5,9Ac_2 and Neu5Ac2en are shown in Fig. 1. The mass fragments of these and other O-acetylated sialic acids are given in Table I. In the spectra of saturated sialic acids the prominent peak is fragment h, which is considered as the base peak, allowing discrimination between different sialic acids and determination of the degree of O-acetylation of sialic acids. Thus, the *m/z* values of fragment h for Neu5Ac, Neu5,9Ac_2, Neu5,7,9Ac_3 and Neu5,7,8,9Ac_4 differ by 42 units each, corresponding to the difference of one O-acetyl residue each (Table I). Similar differences are exhibited by the other fragments of these sialic acids. The fragmentation patterns of the mono-O-acetylated sialic acids Neu4,5Ac_2, Neu5,7Ac_2 and Neu5,9Ac_2 are qualitatively identical and only slight differences in the intensities of the peaks are seen. Therefore, the position of the O-acetyl groups cannot be elucidated by this method. However, different O-acetylated sialic acids separate well on HPLC, thus allowing the localization of the position of O-acetyl groups indirectly by the use of standards. Neu5Ac and Neu5Gc can readily be discriminated by their mass fragmentation patterns, all *m/z* values differing by 16 mass units. The simplicity of the mass spectrum of Neu5Ac2en showing only two ions at *m/z* = 292 $[M + H]^+$ and 274 (292 − 18) is remarkable (Fig. 1).

Fragmentation reactions induced by chemical ionization of saturated sialic acids and of Neu5Ac2en, which are assumed to lead to the fragments of Fig. 1 and Table I, are shown in Figs. 2 and 3, respectively. This fragmentation pattern can be explained as follows. As most fragments of one sialic acid differ by 18 mass units, it is concluded that the $[M + H]^+$ ions lose several water

TABLE I

MASS FRAGMENTATION OF UNDERIVATIZED SIALIC ACIDS SUBJECTED TO COMBINED HPLC—MS

Compound analysed	Characteristic ions (a—j)* at *m/z*									
	j	i	h	g	f	e	d	c	b	a
Neu5Ac	168	186	204	222	246	256	264	274	292	310
Neu5Ac2en	—	—	—	—	—	—	—	—	274	292
Neu5Gc	184	202	220	238	262	272	280	290	308	326
Neu4,5Ac_2 / Neu5,7Ac_2 / Neu5,9Ac_2	210	228	246	264	—	—	306	316	334	352
Neu5,7,9Ac_3	—	—	288	306	—	—	348	358	376	394
Neu5,7,8,9Ac_4	—	—	330	348	—	—	390	400	418	436

*Ion a corresponds to $[M + H]^+$ and fragments b—j derive from ion a by the loss of water (18 mass units), carbon monoxide (28 mass units) or ketene (42 mass units). See also Figs. 2 and 3.

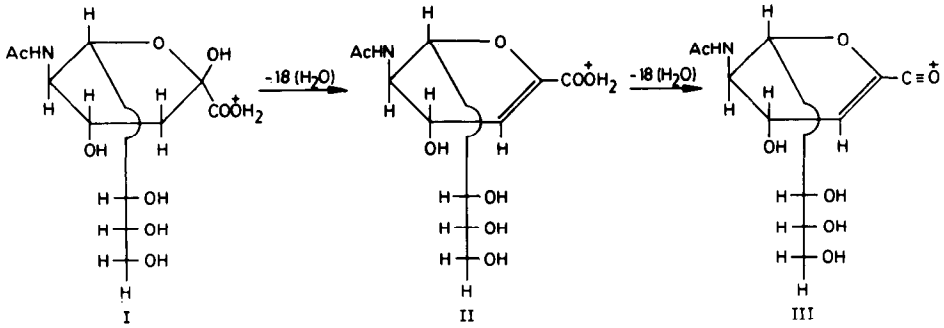

Fig. 3. Mass fragmentation pathway proposed for chemically ionized, positive ions of underivatized Neu5Ac (ring form) and Neu5Ac2en by combined HPLC—MS. I, $[M + H]^+$, m/z = 310 for Neu5Ac; II, $[M + H]^+$, m/z = 292 for Neu5Ac2en, or $[M + H - H_2O]^+$, m/z = 292 for Neu5Ac; III, $[M + H - H_2O]^+$, m/z = 274 for Neu5Ac2en, or $[M + H - 2H_2O]^+$, m/z = 274 for Neu5Ac.

molecules until a stable ion is formed. Fragment h, for example, with m/z = 204 for Neu5Ac, seems to be a most favourable ion as it is the promiment peak. This is also true for the corresponding fragments h of Neu5Gc (m/z = 220) and Neu5,9Ac$_2$ (m/z = 246) (Fig. 1). A scheme for the formation of the h fragment from Neu5Ac including the elimination of two water molecules and of CO and $CH_2{=}C{=}O$ groups is shown in Fig. 2. It is imaginable that for Neu5Ac the h fragment (m/z = 204) can also be formed by the removal of the acetyl group (43 mass units) from the nitrogen atom of fragment b, in addition to the carboxyl residue (45 mass units; not shown in Fig. 2). Assuming this fragmentation pathway for Neu5Gc, the removal of the glycolyl group (59 mass units) and of the carboxyl group from fragment b would result in a h fragment of m/z = 204. As such a peak is missing in the mass spectrum of Neu5Gc and the prominent h fragment is m/z = 220 (Fig. 1), the N-acyl groups seem not to be removed during the fragmentation of sialic acid (Fig. 1). Thus, the formation of the h fragments appears only to be possible by the removal of the C-1 to C-3 part of the nine-carbon chain of sialic acids according to the fragmentation pattern shown in Fig. 2. The assumption of the removal of a H_2O molecule between C-5 and C-6 from $[M + H]^+$ or $[M + H - H_2O]^+$ ions resulting in fragment c as depicted in Fig. 2 is supported by the observation that sialic acids with O-acetyl groups at C-4, C-7, C-8 or C-9 form ion c without losing the acetyl group (see Table I). The fragments discussed so far, with the exception of the upper form of fragment b of Fig. 2, can be explained by the existence of the open-chain configuration of the sialic acids. The existence of this form of free, underivatized sialic acid has been demonstrated by proton-NMR spectoscopy [2]. Therefore, as expected, the fragmentation of underivatized sialic acid is different from the well known fragmentation scheme observed for the electron-impact mass spectra of the methyl ester, per-O-trimethylsilyl ether derivatives of sialic acids having as glycosides ring structures [1].

As part of the Neu5Ac and the other saturated sialic acid molecules in aqueous solution are present in ring form, formation of fragment b from Neu5Ac may also occur as shown in Fig. 3. The first fragment formed

($[M + H - H_2O]^+$, m/z = 292) corresponds to Neu5Ac2en and the second ion represents $[M + H - 2H_2O]^+$. However, comparison of the peak intensities (fragments b and h of Neu5Ac) indicates that fragmentation of Neu5Ac mainly occurs in the open chain form. Neu5Ac2en, which only exists in ring form, shows a quite different fragmentation behaviour when compared with that of saturated sialic acids (Fig. 1). It forms almost exclusively the $[M + H]^+$ and $[M + H - H_2O]^+$ ions (Fig. 3).

The trisaccharide sialyllactose was also analysed by HPLC—MS without prior derivatization. The mass spectrum for sialyl-α(2-3)-lactose is presented in Fig. 4. It exhibits the fragments for lactose ($[M + H]^+$, m/z = 343), and for galactose or glucose (m/z = 180), and also shows the typical fragmentation pattern for Neu5Ac. However, the molecular ion peak for Neu5Ac is missing in this spectrum.

In conclusion, the analytical data presented demonstrate that the HPLC—MS technique is useful for the structural analysis of free or glycosidically bound sialic acids. The method does not require derivatization steps and amounts of 1—10 μg sialic acids can rapidly be analysed.

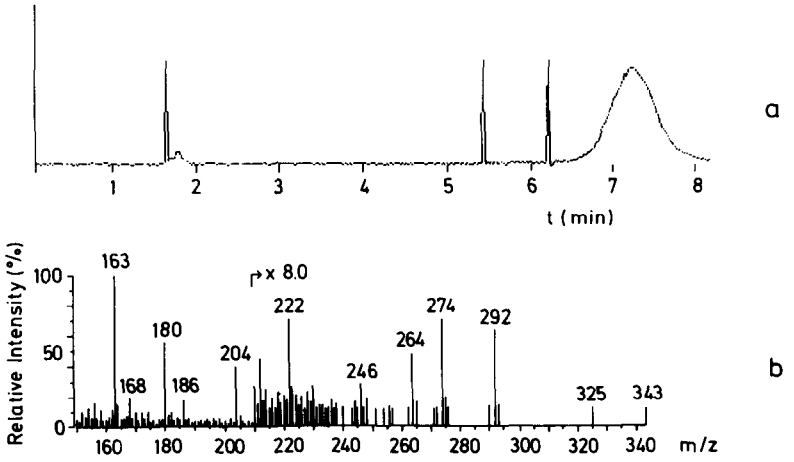

Fig. 4. Analysis of underivatized sialyl-α(2-3)-lactose by HPLC—MS in the positive-ion mode. (a) Total-ionization chromatogram (sharp peaks are due to air bubbles). (b) Mass spectrum; explanation of fragments: m/z = 343, $[\text{lactose} + H]^+$; m/z = 325, $[\text{lactose} + H - H_2O]^+$; m/z = 292, 274, 264, 246, 222, 204, 186 and 168 are typical fragments of Neu5Ac; $[M + H]^+$ of Neu5Ac is not visible; m/z = 180 represents glucose or galactose.

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