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Review

Separation methods for sialic acids and critical evaluation of their biologic relevance

Fotini N. Lamari, Nikos K. Karamanos*

Department of Chemistry, Section of Organic Chemistry, Biochemistry and Natural Products, Laboratory of Biochemistry, University of Patras, 261 10 Patras, Greece

Abstract

Sialic acids are biosynthesized by almost all organisms as a 9-carbon carboxylated monosaccharide and are integral components of glycoconjugates. More than 40 naturally occurring sialic acid derivatives of the three main forms of sialic acids, the *N*-acetyl- and *N*-glycolylneuraminic acid and 2-keto-3-deoxy-nonulosonic acid have been identified. Due to the great importance of sialic acids as key mediators in a plethora of cellular events, including cell–cell recognition and cell–matrix interactions, their analysis in biologic samples is useful for a deeper understanding of the various (patho)physiological processes and of value in disease diagnosis and monitoring. In this review we summarize the methodology developed to isolate and liberate sialic acids from biologic samples as well as the chromatographic, electromigration and hyphenated techniques available for their separation and analysis. A critical evaluation of the biological relevance of the results obtained by analyzing sialic acids in biologic samples is also presented.
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*Corresponding author. Fax: +30-610-997-153.
E-mail address: n.k.karamanos@upatras.gr (N.K. Karamanos).

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1. Introduction to sialic acid structure and function

1.1. Structure of monomeric sialic acid forms

Guntar Blix, Ernst Klenk and Alfred Gottshalk were the pioneers in the field of sialic acid research and established their nomenclature rules in the 1960s [1–3]. Sialic acid is the generic name given to a family of acylated derivatives of a 9-carbon carboxylated monosaccharide (Fig. 1). The carboxyl group at C_1 confers the molecule a negative charge under physiological conditions (pK_a 2.2). Progressive improvements in analytical techniques in recent years have unraveled over 40 different modifications [4]. Substituents on the C–5 can be either an amino, an acetamido, a glycolyl, or a hydroxyl group, thus defining the four major types of sialic acids: neuraminic acid, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (KDN) [5,6]. Neuraminic acid does not exist in nature: only the other forms exist. Neu5Ac and Neu5Gc are the most abundant forms of sialic acids. Neu5Gc is widespread in the deuterostome animals with the notable exception of *Homo sapiens* due to a frameshift mutation in the human gene encoding CMP-Neu5Ac hydroxylase leading to an inactive protein [7–10]. Traces of Neu5Gc have been detected in healthy human tissues and in human cancer tissues, cell lines and sera of cancer patients [8,11]. The natural occurrence of KDN was first reported in 1986 and ever since its ubiquitous presence from bacteria to human has been shown [12]. Although KDN is a minor component in mammalian tissues and cells, elevated expression of KDN in fetal cord blood and ovarian cancer cells has been reported [12].

The great structural diversity in the sialic acid family originates from substitutions of the hydroxyl groups present on carbons 4, 7, 8 and 9 of the four main sialic acid types by methyl (Me), acetyl (Ac), lactoyl (Lt), sulfate (S) or phosphate groups (Fig. 1). The commonest modification is the esterification of the hydroxyl groups at carbons 7, 8, 9 and rarely at C_4 by acetyl groups (*O*-acetylation). Other modifications include introduction of lactoyl or phosphate groups to C_9 and introduction of methyl and sulfate groups to C_8. Furthermore, unsaturated sialic acids or sialic acids with internal anhydro linkages have been discovered [4].

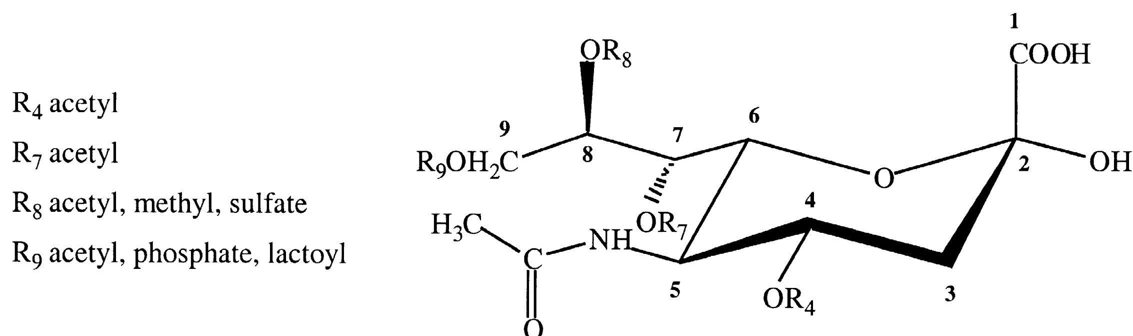

Fig. 1. Chemical structure of the main representative of the sialic acid family, Neu5Ac (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactopyranos-1-onic acid) and the commonest modifications. Neu5Gc contains the glycolyl group bound to the C_5 amino group instead of the acetyl group.

1.2. Location and linkages of sialic acids in glycoconjugates

Sialic acids occupy strategic location on the outermost part of the carbohydrate moieties of membrane and secreted glycoconjugates (glycolipids, glycoproteins and lipopolysaccharides). They are usually the terminal moieties in the glycan chains and are glycosidically linked via their hydroxyl group at C_2 to position 3 or 6 of the penultimate sugar or to C_8 of another sialic acid residue. The latter event is rather seldom and the most frequent penultimate sugars are galactose residues. However, linear homopolymers (*n* about 8 to over 100) of sialic acid attached to glycoproteins have been identified in a wide variety of living species from bacteria to human [12]. The occurrence of two polysialylated glycoproteins was demonstrated by our research group in the sea urchin shell, which is a very useful model for studying the participation of carbohydrate-containing macromolecules in developmental regulation [13]. These macromolecules contain sulfated poly(sialic acid) polysaccharides of similar chemical composition that are *N*-glycosidically linked to different core proteins. In general, the importance of these structures is demonstrated by the finding that poly(sialic acid) chains are also expressed in the embryonic form of neural cell adhesion molecule in a developmentally regulated manner.

1.3. Properties and biologic functions associated with sialic acids

General functions of sialic acid residues at the cell surface, like binding and transport of positively charged molecules, attraction/repulsion of cells and molecules as well as their antiproteolytic effect in glycoproteins are attributed to their physicochemical properties, i.e. electronegativity and hydrophilicity. Moreover, their particular localization provides them accessibility reflected in their potential to regulate a multitude of cellular and molecular recognition phenomena. They serve as antigenic determinants or components of receptors for a variety of molecules and cells, such as hormones and cytokines, toxins, viruses, bacteria and protozoa [5]. They also serve as ligands for receptors, such as plant lectins, selectins and siglecs, mediating a variety of cell–cell and cell–matrix processes in immune response and cell migration. A well-known example is the significant effect of interactions between endothelial selectins and leukocyte sialylated Lewis antigens on the adhesion of white blood cells to endothelia.

Apart from being biologic targets themselves, they often mask other recognition sites [5,6]. Sialic acids render cells as "self" preventing activation of the immune system. Microorganisms/malignant cells have exploited this phenomenon by oversialylation of their cell surface, thus being protected from humoral and cellular immune defense systems. On the other hand, desialylation leads to recognition by galactose-specific lectins, thus promoting targeting of molecules and cells to specific sites, e.g. targeting of desialylated serum glycoproteins to hepatocytes for their removal from circulation. Bacterium or virus induced desialylation may lead to the production of autoantibodies implicated in chronic diseases, such as glomerulonephritis. Their involvement in all these cellular processes makes obvious their involvement in tumor biology and abnormal glycosylation patterns are very common in human and animal neoplasias [11,14,15].

O-acetylation significantly alters the physicochemical properties of the parent molecule and can therefore modify and/or create new biologic functions [16]. *O*-acetylated sialic acids show an interesting cell and molecule specificity, have a developmentally regulated expression and may be re-expressed in malignancies as oncofetal antigens. Knowledge on the function of 4-*O*-acetylated sialic acids is limited and restricted to the resistance to sialic acid degrading enzymes. More information is common about 7-, 8- and 9-*O*-acetylation [17,18]. It is noteworthy that *O*-acetyl esters from positions 7 and 8 spontaneously migrate to position 9 unless already substituted. 9-*O*-acetylation mediates cellular cross talk and thus tissue differentiation and provides non–immune protection to colonic mucosae.

1.4. Methods for determination of sialic acids

Spectrophotometric and enzymic reactions, antibodies, lectins and viruses have all been used to determine either the total sialic acid content or specific sialic acid types [17,18]. Colorimetric meth-

ods using orcinol, resorcinol, periodic/thiobarbituric acid and periodic acid/methyl-3-benzothiazolone-hydrazone have been widely used throughout the years [19]. Common limitations of these procedures are their inability to differentiate the types of sialic acid and the requirement for purified samples to avoid interference from other lipid contaminants. Various modifications of the acylneuraminate pyruvate lyase system have been reported for the enzymic determination of sialic acids, while antibodies of varying degree of specificity have proved an indispensable tool in a number of studies using immunofluorescence, immunohistochemistry and even immunostaining of thin-layer chromatograms. Lectins of animal and plant origin have also been used as probes of sialic acid residues and their various naturally occurring derivatives. On the other hand, viruses had a very significant impact on sialic acid analysis. For example, influenza C virus has a surface protein with both hemagglutinin and esterase activities: the former allows the virus to bind to peripheral proteins bearing 9-*O*-acetylated sialic acids and the latter to cleave the bound sialic acid residue. At inhibitory conditions for the esterase activity either the virus particles or chimeric products of this protein are widely used [17,18].

However, steady refinement of analytical techniques, such as high-performance liquid chromatography (HPLC) and gas-chromatography (GC) combined with mass spectrometry (MS), nuclear magnetic resonance (NMR) spectrometry and recently capillary electrophoresis (CE), has allowed for more definite elucidation of the structures of sialic acids with different acylation patterns and more sensitive determination. Aim of this article is to review on the methods to liberate sialic acids from biologic samples and the advances in the chromatographic, electrophoretic and hyphenation procedures for sialic acid analysis in the recent years. We also attempted to present a critical evaluation of the biologic relevance of the analytical results based on our experience in the field.

2. Methods to liberate sialic acids from biologic samples

In spite of the variety of analytical methods available for sialic acids, their analysis in biologic samples remains a difficult task. Critical step in sialic acid analysis is their liberation from the biologic sources, which entails isolation of the sialylated macromolecules and/or removal of the sialic acid residues from the glycoconjugates. For isolation of sialylated macromolecules from blood serum and tissue specimens, we have proposed a simple procedure. Isolation from serum includes a precipitation step with two volumes of saturated ammonium sulfate, pH 10.0, and subsequent gel chromatography on pre-packed Sephadex G-25 columns, where the void volume fraction is collected [20]. Approximately 98.5% of total sialic acids are recovered by this procedure as de-*O*-acetylated derivatives [20]. Alternatively, the direct hydrolysis of serum without any prior treatment has been reported [21]. Isolation from tissue specimens is performed with homogenization with water at 0 °C and following centrifugation the water-soluble sialic acid macromolecules are almost quantitatively recovered in the supernatant [20].

Liberation of sialic acids from the purified samples is achieved either by hydrolytic procedures or treatment with specific enzymes. A number of sialidases or neuraminidases (EC 3.2.1.18) from various microorganisms have been characterized (for a thorough review refer to that of Traving and Schauer [5]). Although their role in microbial pathogenesis is not fully understood, their catalytic action has been used for the release of sialic acid residues from the non-reducing terminal of various glycoproteins and glycolipids. Many sialidases are commercially available and their specificity depends on *O*-acetylation, the occurrence of *N*-glycolyl groups and the type of the glycosidic linkage. The sialidase from *A. ureafaciens* has a broad linkage specificity and thereof has been extensively used for the non-selective cleavage of non-reducing terminal sialic acid from *N*- and *O*-glycans from both glycoproteins and glycolipids. Despite their profound advantages, the action of sialidases is limited to the terminal residues and is dependent on several factors ranging from the substitution pattern to the sample composition, which are not easily controlled in biologic samples.

The low cost, ease and effectiveness of hydrolytic procedures have established them the method of choice in many laboratories, even though they are always associated with some destruction of these carbohydrates. Acidic hydrolysis with 2 *M* trifluoroacetic acid (TFA) at 80 °C ensures the hydrolytic

cleavage of all sialic acid linkages, deacylation and decarboxylation of these residues [22]. Thus, the total sialic acid content can be determined by measuring the amino-product generated by this procedure. The optimum hydrolysis time is 2 h, whereafter there is some destruction depending on hydrolysis time. Here, it should be stated that it is imperative in all hydrolytic procedures to treat the sialic acid standards under the same conditions. The amount of hexosamines liberated from glycoproteins with this procedure is very low (<10%) [22]. In order to avoid the interference of neutral hexoses and hexosamines, the dried hydrolysates are subject to ion-exchange chromatography on Dowex 50×8 (H^+ form) and Dowex 1×8 ($HCOO^-$) columns [20]. This procedure ensures the unobstructed derivatization and HPLC analysis, although we recently showed that just an ultrafiltration step on centricon-3 membranes (cut-off 3-kDa) to remove macromolecules after hydrolysis is enough for sialic acid determination with CE [23].

Nevertheless, the aim of most hydrolytic procedures is the recovery of sialic acid residues in their two basic types, Neu5Ac and Neu5Gc. At mild acid conditions (25–100 m*M* of most inorganic acids) sialic acids retain their carboxyl and *N*-acyl groups. The main concern in hydrolytic procedures is the minimization of carbohydrate destruction and the maximization of sialic acid release and many studies have focused on the optimization of conditions. Hydrolysis with 25 m*M* hydrochloric acid or TFA at 80 °C for 2 h has been suggested after a careful study of the various hydrolytic conditions [24]. Although hydrolysis with these acids for 2 h results in losses of ca. 20%, this time ensures complete hydrolysis of sialic acid linkages irrespective of the glycoprotein used [24]. Our experience shows that de-*O*-acetylation at these conditions is complete, but if this is doubted for special reasons then a complementary alkaline treatment is recommended. Alkaline treatments (4 *M* NH_4OH at 25 °C for 4 h; 0.1 *M* NaOH at 37 °C for 30 min; 2 *M* NH_4OH at 25 °C for 6 h) induce saponification of *O*-acetyl esters [17]. Hydrolysis with 25 m*M* sulfuric acid is also associated with minor losses but these hydrolysates are difficult to lyophilize [25]. Ogawa et al. [26] suggested incubation at 80 °C for 1 h in vacuum in 0.01 *M* hydrochloric acid with removal of the hydrochloric acid by evaporation without decomposing the sialic acids. This was confirmed by Shaw et al. [27] since they reported overall mean recoveries of about 98%. Hydrolysis in microwaves (only 10 min required) with 2 *M* CH_3COOH has been suggested by Lagana et al. [28].

Slightly modified hydrolytic conditions are necessary for glycolipids. Treatment with 1% acetic acid at 70 °C for 60 min has been suggested for the complete release of KDN from deacetylated lipopolysaccharide (LPS) with concomitant destruction of 15% [29]. These authors demonstrated that treatment at higher temperatures usually employed results in significant losses. Mattoo and Roseman [30] optimized the hydrolytic conditions for the quantitative release of Neu5Ac and Neu5Gc from gangliosides and suggest hydrolysis in 0.1 N H_2SO_4 containing 0.2% SDS at 85 °C for 60 min.

For a thorough study of the biologic importance of *O*-acetylation, hydrolytic conditions should be optimized for their complete release as substituted types. Varki and Diaz [31] have pointed out an additional problem; the migration of *O*-acetyl groups from the 7 or 8 positions to position 9, particularly when the pH is above 6 or below 3. Strong acids (H_2SO_4 or HCl) should certainly be avoided. Formic acid induces less destruction, but the release is incomplete. However, a three-round formic acid hydrolysis (pH 2.0) has proved efficient for the release of Neu4,5Ac [32]. Treatment with 2 *M* acetic or propionic acid at 80 °C for 2–3 h allows complete release of 7-, 8- and 9-*O*-acetylated sialic acid with minimal destruction [31,33]. Stehling et al. [34] have reported that the loss of acetyl groups attached to the hydroxyl groups at C_7, C_8 or C_9 was less than 7% when the glycoconjugates were treated with 2 *M* acetic acid at 80 °C for 1 h.

In summary, acid hydrolysis is an effective means to liberate sialic acid residues, but balance between release and destruction should be optimized. Enzymic treatment with neuraminidase may be used to avoid destruction. However, only a neuraminidase treatment may not release all the sialic acids from a sample and, therefore, the acid treatment will provide the more accurate answer and/or may aid in the development of an optimized neuraminidase treatment [35]. Neuraminidase treatment may provide an accurate assessment of a sample's sialic acid content on the condition that *O*-acetyl groups have been previously removed and the enzyme has broad

linkage specificity. The choice of the appropriate method also depends on the subsequent steps for analysis; e.g. if the sample is derivatized after cleavage, care must be taken so that the solution components do not interfere with the derivatization reaction.

3. Chromatographic, hyphenation and electromigration procedures

There are two levels of sialic acid analysis: analysis of sialic acids as monosaccharide constituents and structural analysis of the glycan chains of glycoconjugates. The latter deals with carbohydrate structural organization which is usually very complicated, due to the presence of many monosaccharides with multiple and diverse substitutions, the numerous possibilities for glycosidic linkage among these, the presence of several different glycosylation sites in a glycoconjugate, and the incomplete biosynthesis or degradation/processing of the glycans. Such an analytical challenge is addressed by a combination of techniques, such as HPLC, GC–MS, LC–ESI MS, NMR, CE and endo- and exoglycosidase digestions. There are excellent reviews covering this area of research [36–38]. This review focuses on the separation techniques for analysis of sialic acids mainly as monosaccharide constituents that can easily be applied in daily laboratory practice to give results of significant biologic relevance.

3.1. Chromatographic and hyphenation procedures

Analysis of sialic acids has been mainly performed by chromatographic methods, liquid and gas chromatography. GC–MS was originally used to characterize many of the *O*-substituted neuraminic acids and the glycosidic linkages usually after permethylation. Recently, a method involving the methyl esterification using diazomethane in the presence of methanol and the formation of volatile derivatives using heptafluorobutyric anhydride was developed [39]. The derivatives were analyzed by GC–MS in the electron impact mode. This technique allowed the separation and identification of a large variety of sialic acids, including different *O*-acylated forms of Neu5Ac, Neu5Gc and KDN and 8-*O*-methylated and 8-*O*-sulfated derivatives, de-*N*-acetylated neuraminic acid, and 1,7-sialic acid lactones [39]. However, HPLC has been more widely used and in this review we will focus on the liquid chromatographic methods.

3.1.1. Direct analysis of sialic acids

Application of liquid chromatographic methods to the analysis of sialic acids without prior derivatization has attracted the interest of many researchers in the last years. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is the most powerful tool for analysis of carbohydrates in general and underivatized sialic acids in particular. In 2000 Rohrer authored an excellent review on the HPAEC–PAD analysis of sialic acids and the interested reader may refer to it [40]. By and large, the solutes are eluted either isocratically with a NaOH/sodium acetate eluent or with a constant NaOH concentration and a gradient of sodium acetate. The highly alkaline conditions used limit the range of sialic acids to the non-substituted ones, Neu5Ac, Neu5Gc and KDN. In an attempt to overcome this problem, Manzi et al. [41] reported the separation of *O*-acetylated sialic acids with an acetic acid/sodium acetate eluent and sodium hydroxide was added to the column effluent for the detection of sialic acids by PAD.

A reversed-phase ion-pair HPLC analysis of five underivatized sialic acids (Neu5Ac, Neu5Gc, Neu5Ac2en, CMP-Neu5Ac and Neu5,9Ac_2) has been reported [42]. Prior to analysis, the hydrolyzed samples were passed through a cartridge Sep-Pak C_{18} and then analysis was performed isocratically on a C_{18} column with an aqueous solution of 60 m*M* Tris–isopropanolamine, as ion-pair reagent, pH 3.5 and UV detection. This method was successfully applied to the analysis of normal and pathological sera from patients with breast, stomach, colon, ovarian and cervix cancers, to normal urine and urine from patient with sialuria and to normal saliva [21]. Alternatively, Neu5Ac and Neu5Gc directly after hydrolysis were separated on a C_8 column using 0.1% (v/v) aqueous formic acid [27]. Quantitative analysis was performed using as internal standard *N*-acetylneuraminic acid methyl ester by positive ion electrospray ionization MS and the response was

linear over the concentration range of 5 to 100 μg/ml [27].

3.1.2. Analysis of sialic acids following derivatization

3.1.2.1. Analysis of de-O-acetylated sialic acids. Derivatization is easy, offers significantly higher sensitivity and selectivity, and has been widely employed. The analysis of the total amount of sialic acid, glucosamine and galactosamine under the same chromatographic conditions was described by Makatsori et al. [22]. Two different hydrolytic procedures of glycoconjugates yield the hexosamines and a deacetylated decarboxylated amino-product representative of total sialic acid content. Following removal of neutral monosaccharides and aminoacids by ion-exchange chromatography, derivatization with *p*-toluenesulfonylchloride (Tos-Cl) yields Tosyl-derivatives, which are completely resolved on a Supelcosil LC_{18} column by isocratic elution using a mobile phase of water–acetonitrile (85:15, v/v). In particular, 25 μl of water, 37.5 μl of Tos-Cl reagent (50 mg of Tos-Cl in 10 ml acetone) and 37.5 μl of triethylamine solution (100 μl triethylamine in 10 ml of acetone) and was heated at 60 °C for 40 min. Excess of reagent and non-tosylated derivatives were removed by passing the mixture through a Sep-Pak C_{18} cartridge. The method shows a linearity range up to 25 nmol of sialic acids and hexosamines, has a detection limit ranging from 6 to 12 pmol, and is easily applied to the analysis of biologic samples [22].

Per-*O*-benzoylation has been extensively and successfully used by our research group for the sensitive determination of Neu5Ac and Neu5Gc [24]. To the dry hydrolysate 100 μl of benzoylation mixture [10% (w/v) benzoic anhydrite–5% (w/v) *p*-dimethylaminopyridine in pyridine] are added and the mixture is heated at 80 °C for 20 min. The reaction is terminated by the addition of nine volumes of water and vigorous shaking. For a complete destruction of the remaining benzoic anhydrite, the mixture obtained was heated for a further 10 min at 80 °C. Excess of reagents and minute amounts of under-benzoylated derivatives are removed by solid-phase extraction on Sep-Pak C_{18}. Per-*O*-benzoylated derivatives are highly UV absorbent and are nicely separated on a Supelcosil LC_{18} column by isocratic elution with 67% (v/v) aqueous acetonitrile. The Neu5Gc-derivative is eluted nearly 10 min later than the Neu5Ac derivative since it carries an extra benzoyl group on the *N*-glycolyl moiety (Fig. 2). The method allows the determination of both sialic acid forms at the picomolar level and the calibration graphs are linear up to 160 nmol. This method has been applied to the quantitative determination of Neu5Ac and Neu5Gc in blood serum, animal and human tissues as well as for disease diagnosis and monitoring [11,14,15,20].

The UV-absorbing tag 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP) (λ_{max}=245 nm) has been used to derivatize reducing sugars in the presence of carbodiimide by a condensation reaction under mild conditions, which does not cause desialylation or desulfation [43]. The bis-PMP derivatives behave

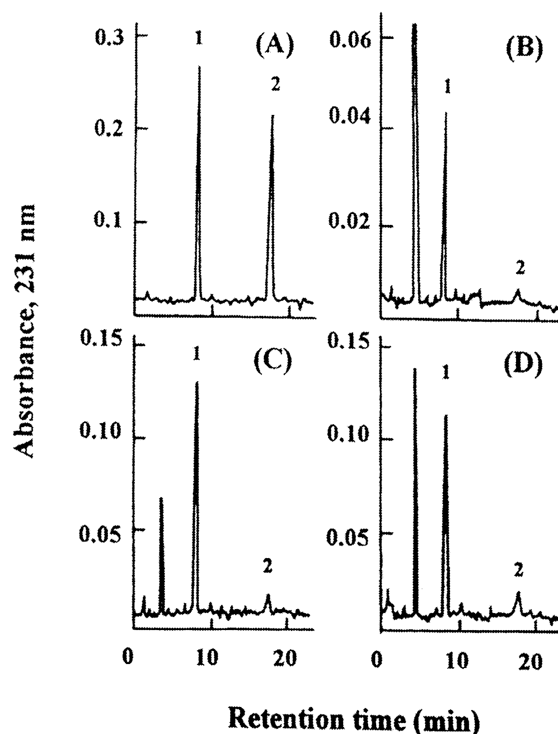

Fig. 2. Separation of Neu5Ac (peak 1) and Neu5Gc (peak 2) after per-*O*-benzoylation on a Supelcosil LC_{18} column by isocratic elution with 67% (v/v) aqueous acetonitrile [11]. HPLC profiles of these sialic acids in a standard mixture (A), sera from a healthy woman and with endometrial cancer (B and C, respectively) and a tissue specimen (D). Reprinted with permission from Elsevier.

like weak anions in aqueous basic solutions, strongly absorb the UV light and can be detected electrochemically. In a study of Fu and O'Neill, sialic acids are converted with neuraminic acid aldolase to their corresponding mannosamine derivatives, which are then separated by reversed-phase HPLC [44]. It was reported that individual sialic acids including Neu5Ac and Neu5Gc are well resolved.

The reversed-phase HPLC analysis of sialic acids after labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) is one of the most widely used highly sensitive methods for the determination of sialic acids [25]. Sialic acids react with DMB to form quinoxaline derivatives in various derivatization conditions. The resultant quinoxaline derivatives show strong fluorescence at 448 nm on irradiating at a 373 nm light. Only an α-keto carboxylic acid group (C_1 and C_2 of the sialic acid residue) is involved in derivatization, while others are intact. Hara et al. [25] first reported the development of reversed-phase HPLC fluorimetric method. Recent studies have experimented with the introduction of internal standards in the method in order to improve the accuracy [45,46]. Hikita et al. [46] optimized the procedure for analysis of Neu5Ac and Neu5Gc in gangliosides and hence used the ganglioside GM3 having *N*-propionylneuraminic acid (NPNA) as an internal standard. Based on this study, Ito et al. [45] synthesized NPNA, which was used as an internal standard successfully (Fig. 3). Both separations are performed on C_{18} columns with isocratic elution with a mobile phase of methanol–acetonitrile–10 m*M* sodium acetate buffer (pH 5.0).

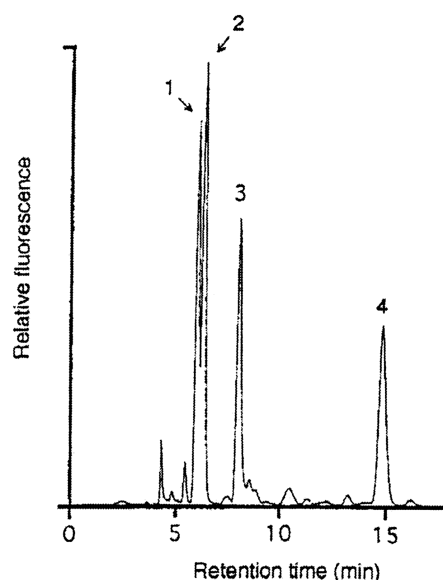

Fig. 3. Chromatogram showing the separation of DMB derivatives of Neu5Ac (peak 3), Neu5Gc (peak 2), KDN (peak 1 and 2) and NPNA (peak 4) on a C_{18} column using methanol–acetonitrile–10 m*M* acetate buffer (pH 5.0) (25:4:91, v/v/v) [45]. Reprinted with permission from Elsevier.

3.1.2.2. Analysis of naturally occurring O-acetylated derivatives of sialic acids. The analysis of *O*-acetylated derivatives of sialic acids has attracted the interest of many researchers. The acidic conditions (acetic acid or sodium hydrogensulfite) used in the DMB derivatization reaction seem to prevent hydrolysis or migration of acetyl groups from one to another hydroxyl group, and this makes it appropriate for the analysis of *O*-acetylated derivatives [25,34,47]. Stehling et al. [34] reported the separation of six different *O*-acetylated sialic acid derivatives by reversed-phase liquid chromatography on a C_{18} column. Quinoxaline derivatives were eluted with a non-linear gradient of two eluents; eluent A is water–acetonitrile–methanol (92:4.5:3.5, v/v/v) and eluent B water–acetonitrile–methanol (84:9:7, v/v/v) [34]. Structural characterization was further achieved with parallel analysis with matrix–assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS).

LC–ESI-MS is a powerful tool for analyzing various members of the sialic acid family. Sialic acids are usually derivatized with DMB and then separated by reversed-phase liquid chromatography. A combination of retention times and spectra characteristics allows definition of the type and position of the various *O*-acetyl substituents. The research group of Professor K. Kakehi has developed a strategy for the simultaneous determination of about 13 members of the sialic acid family with differences in the substitution with *N*- or *O*-acetyl, glycoyl and sulfonic ester groups [47,48] (Fig. 4). Sialic acids are directly derivatized with DMB after their release by acid hydrolysis, and are separated on an octadecyl silica column with a linear gradient from a mixture of methanol–acetonitrile–water (14:7:79, v/v/v) to the same mixture (14:11:75, v/v/v) (Fig. 4). De-

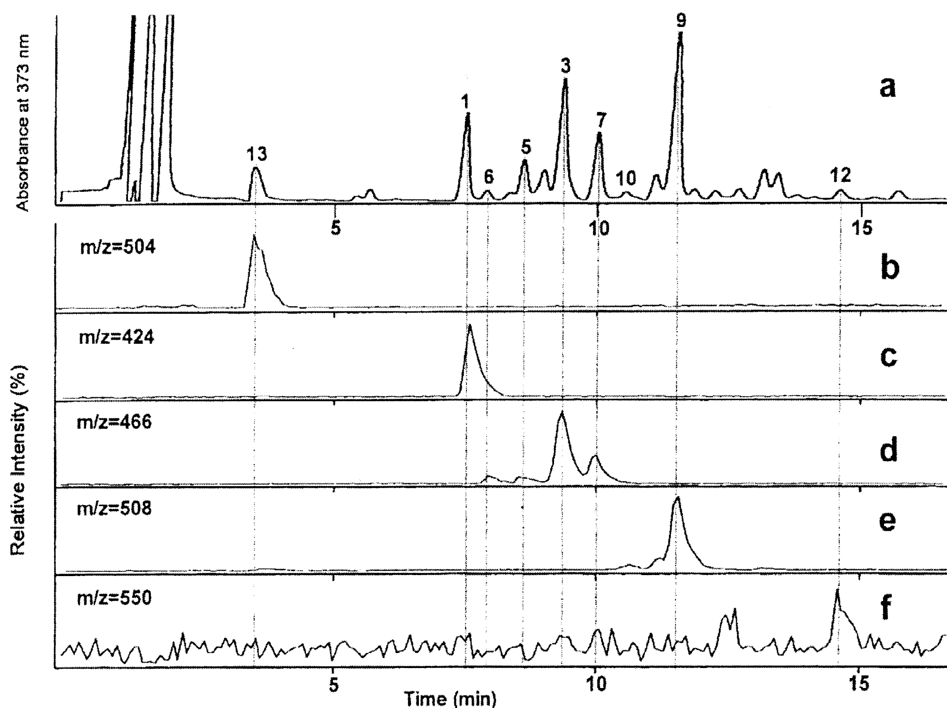

Fig. 4. LC–ESI-MS of sialic acids from rat sublingual glands as their DMB derivatives and detected by measurement of the absorbance at 373 nm (a) and monitored at *m/z* 506 (b), 426 (c), 468 (d), 510 (e) and 552 (f). Peaks 1, Neu5Ac; 3, Neu5,9Ac$_2$; 5, Neu5,8Ac$_2$; 6, Neu5,7Ac$_2$; 7, Neu4,5Ac$_2$; 9, Neu5,7,9Ac$_3$; 10, Neu5,8,9Ac$_3$; 12, Neu5,7,8,9Ac$_4$;13, Neu5Ac8S [47]. Reprinted with permission from the American Chemical Society.

tection was performed either with a fluorimonitor or with connection to an ESI-mass tandem spectrometer. LC–ESI-MS helped the structural confirmation of the HPLC peaks (Fig. 4). Thus, characteristic distributions of sialic acids in the tissues of mice and rats were revealed [47]. Klein et al. [49] identified 28 different sialic acids including the following new species: Neu5Gc9Lt (bovine subamaxillary mucin), anhydro derivatives of Neu5Ac other than the 4,8-anhydro (horse serum hydrolyzates), KDN5(7)Ac and KDN5(7),9Ac2 (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*). These results showed that LC–ESI-MS is a powerful tool for studying the sialic acid diversity, and identification of small amounts of unexpected sialic acids or new members of their family.

3.2. Electromigration procedures

Capillary electrophoresis (CE) is the most modern analytical electromigration technique successfully used for the analysis and structure characterization of carbohydrates (for a state-of-the art on CE see the review by Lamari and Karamanos [50]). The analysis of the monosaccharide constituents of glycoconjugates has been the focus of intense research in the last years and there are a number of excellent reviews [51–56]. The commonest mode of CE used is capillary zone electrophoresis (CZE). In CZE, the separation mechanism is based on differences in charge–mass ratio. In the uncoated fused-silica capillary used, the electroosmotic flow (EOF) of buffer will move all analytes, in the normal polarity mode, towards the negative electrode (cathode). Positively charged analytes migrate according to the vector sum of their electrophoretic mobility (EM) and EOF. In the case of anions, however, EOF and EM counteract

each other and the apparent migration depends on the net difference between the two driving forces. At acidic pH, EOF is so low that it cannot overwhelm the EM of anions. In that case, one must reverse the polarity, i.e. by injecting the sample at the cathodic end of the capillary. The separation conditions used for CE analysis of sialic acids are summarized in Table 1.

3.2.1. CE analysis of non-derivatized sialic acids

Most carbohydrates do not possess ionizable groups, i.e. phosphate, sulfate and carboxyl, and their hydroxyl groups are ionized only in extreme alkaline conditions (pH>11). Sialic acids are privileged in that aspect, due to the presence of carboxyl groups. However, derivatization with charged molecules and formation of complexes with borate anions are usually employed to impart higher charge density. Schmitt-Kopplin et al. [57] have theoretically studied the formation of bidentate borate esters. Calculation of the association constants helped them develop a CZE method for separating carboxylated monosaccharides and their 1,4-lactones at neutral pH. Moreover, Dong et al. [58] have reported the separation of underivatized Neu5Ac and Neu5Gc in a 50 m*M* $Na_2B_4O_7$–50 m*M* Na_2HPO_4 buffer, pH 8.95 at 10 kV. Albeit the resolution was not satisfactory, the method could be applied to determination of Neu5Ac in blood sera. Detection was performed at 195 nm and the reported detection limit of Neu5Ac is 9.6 μ*M* (or 39 fmol) [58].

Sialic acids, like all carbohydrate molecules, lack chromophores, and thus indirect detection methods are often employed in order to avoid complex

Table 1
CE methods and operating conditions used for determination of sialic acids

Analytes	Operating conditions (capillary type, operating buffer, voltage, wavelength)	Refs.
Direct analysis		
Neutral, acidic and aminosugars Sugar alcohols	UFS, 20 m*M* 2,6-pyridinecarboxylic acid–0.5 m*M* CTAB (pH 12.1), −25 kV, UV 350 nm with a reference at 275 nm.	[59]
Acidic monosaccharides and their corresponding 1,4-lactones	UFS, 600 m*M* borate (pH 7.1) 20 kV, 200 nm	[57]
Neu5Ac and Neu5Gc	UFS, 50 m*M* borate–50 m*M* Pi (pH 8.95) 10 kV, 195 nm	[58]
Analysis after derivatization		
Sialic acids	UFS, 25 m*M* borate (pH 10.0) 750 V/cm, Ar-ion LIF detector (9-AMAC derivatives)	[61]
Neutral, aminosugars and sialic acids	UFS, 120 m*M* borate (pH 10.2) 25 kV, Ar-ion LIF detector (APTS derivatives)	[62]
Neutral, aminosugars and acidic monosaccharides	300 m*M* borate (pH 10.5) 20 kV, 260 nm (2-AMAC derivatives)	[63]
Acidic monosaccharides	PVA coated, 300 m*M* acetate (pH 5.0) −20 kV, He–Cd LIF detector (ANDSA derivatives)	[64]
Neu5Ac	UFS, 50% (v/v) acetonitrile in 25 m*M* phosphate (pH 3.5) 30 kV, UV 231 nm. (per-*O*-benzoylated derivatives)	[23]
Aminosugars, neutral and acidic monosaccharides	UFS, 200 m*M* borate (pH 10.5) containing 10% methanol, 15 kV, 490 nm and Ar-ion LIF detector (NBD derivatives)	[65]

Abbreviations: UFS, uncoated fused-silica; Pi, phosphate.

derivatization procedures with variable product yield. Soga and Heiger [59] have reported the simultaneous determination of acidic monosaccharides including sialic acids, neutral monosaccharides, aminosugars and sugar alcohols within 30 min, using 2,6-pyridine carboxylic acid, pH 12.1, as the UV marker ion and cetyltrimethylammonium bromide for EOF reversal. The detection limits ranged from 20 to 70 μ*M* and the method was easily applied to composition analysis of glycoproteins.

3.2.2. CE analysis of sialic acids using various derivatization schemes

Derivatization with UV-absorbing and fluorescent molecules results in significant gains for both the separation efficiency and the detection sensitivity. Derivatization has the additional advantage of enhancing the selectivity of analysis since detection is performed at wavelengths where most of the naturally occurring macromolecules do not absorb. The most widely used derivatization scheme is reductive amination. The carbonyl group of a reducing sugar reacts with the amino-group of the label and forms a Schiff base, which is reduced with sodium cyanoborohydride to a stable secondary amine. Various UV-absorbing and fluorescent molecules have been used (see Table 1). According to Kakehi et al. [60], although aminobenzene derivatives substituted at 2- and 4- positions yield derivatives with high fluorescence intensities and molar absorptivities, 3-substituted aminobenzene derivatives (3-aminobenzamide and 3-aminobenzoic acid) show the highest reaction efficiencies, allowing thus mild derivatization conditions, which do not destruct sialic acids.

Guttman [61] used the fluorescent tag 9-aminoacridone (9-AMAC) for the determination of sialic acids. Chen et al. [62] have addressed the problem of the low yield of sialic acid derivatization with APTS by introducing treatment of the hydrolysates with neuraminic acid aldolase (EC 4.1.3.3). Neu5Ac was converted quantitatively to *N*-acetylmannosamine, which was then derivatized with APTS in the same manner as the other monosaccharides [62] (Fig. 5). Previous experiments in our laboratories have showed low yields of derivatization in non-aqueous conditions after enzymic treatment, probably due to the presence of salts, which hinder targeting of the reactants. The quantitative derivatization in the study of Chen et al. might be explained by the fact that APTS derivatization proceeded in a solution of 33% THF and 66% citric acid aqueous solution. The detection limit for Neu5Ac was 100 pmol. In addi-

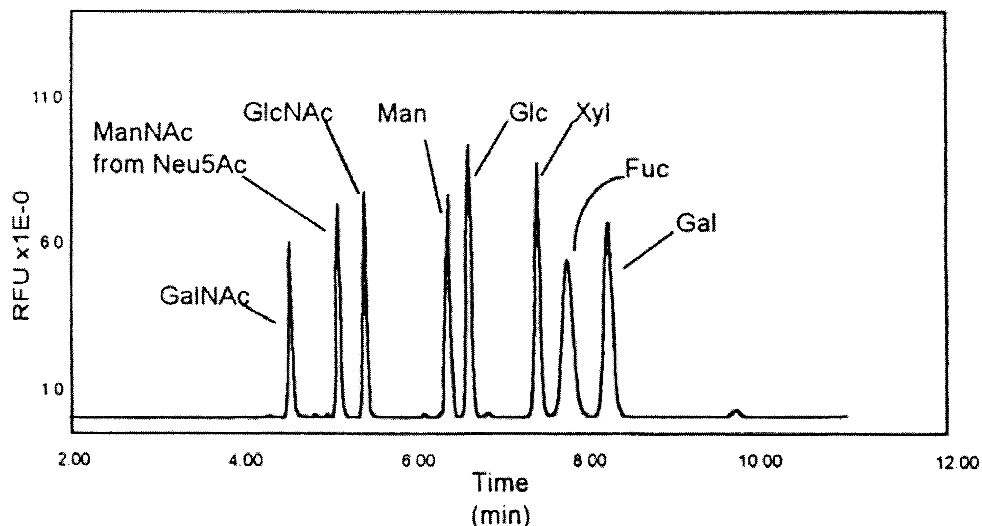

Fig. 5. Electropherogram of APTS derivatives of neuraminic acid aldolase-treated mixture of Neu5Ac and seven aldoses separated in an alkaline borate buffer [62]. Neu5Ac is converted quantitatively to *N*-acetylmannosamine (ManAc). GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; Glc, glucose; Xyl, xylose; Fuc, fucose; Gal, galactose. Reprinted with permission from Oxford University Press.

tion to these studies, Che et al. [63] have studied in detail and optimized the derivatization reaction of sialic acid with 2-AMAC. The AMAC-derivative of Neu5Ac was unstable; nonetheless, the suggested method was successfully applied to the quantitative determination of sialic acid in various glycoproteins with detection limit of 1 μM (or 35 fmol) [63]. Surprisingly, these detection limits are similar to those reported by Dong et al. [58] who analyzed Neu5Ac and Neu5Gc without any prior derivatization, but derivatization is advantageous in avoiding interference from other sample components.

Mechref et al. [64] introduced a different derivatization scheme for sialic acids in 1997. This scheme involves condensation of the carboxylic acid group of Neu5Ac with the amino group of 7-amino-naphtalene-1,3-disulfonic acid (ANDSA). Mechref et al. [64] thoroughly studied the effect of ionic strength on the resolution of ANDSA-derivatives of uronic, aldonic and sialic acids at zero EOF conditions and showed that high ionic strengths are preferable in some separation problems. Recently, Honda et al. [65] introduced a novel derivatization procedure for reducing sugars. Reducing sugars are converted to *N*-methylglycamines in the presence of dimethylamine–borane complex at pH 4.5 and 40 °C. The resultant *N*-methylglycamines were labeled with 7-nitro-2,1,3-benzoxadiole and the derivatives were sensitively determined either by UV–Vis at 490 nm or by a laser-induced fluorescence (LIF) detector using an Ar-ion laser resulting in very high detection sensitivity (nanomolar concentrations) [65]. The main advantage of this scheme is that under the mild conditions of the derivatization, sialic acids are not destructed or released from sialylated glycans [65].

We recently employed the well-known per-*O*-benzoylation procedure for the conversion of Neu5Ac to a highly UV-absorbing molecule [23]. This derivatization scheme was previously used for the successful analysis of sialic acids by HPLC. Analysis of these derivatives by CZE was performed using the operating buffer 25 m*M* phosphate, pH 3.5, containing 50% (v/v) acetonitrile as organic modifier, at 30 kV, and detection of the per-*O*-benzoylated Neu5Ac at 231 nm. The method showed excellent repeatability (RSD$<$1.98%) and a linearity range from 5 μg/ml to 5 mg/ml with a detection limit of 2 μM. The method was successfully applied to micro-analysis of human α_1-acid glycoprotein and blood serum samples.

4. Critical evaluation of the biologic relevance of the analytical results

4.1. Neu5Ac and Neu5Gc in biologic fluids of healthy individuals

The presence of sialic acids in blood serum has been thoroughly studied. In the majority of studies measurement of total sialic acid is performed by determination of Neu5Ac after de-*O*-acetylation, since it is the major or only *N*-acyl derivative. Although other studies have shown the absence of Neu5Gc in serum of healthy humans [58], Neu5Gc was determined after per-*O*-benzoylation with HPLC as a minor component of serum glycoproteins (about 8% of total sialic acids) [11]. The presence of Neu5Gc in hydrolysates of human serum has been earlier shown by paper chromatography [66]. A GC–MS study reported very low amounts of glycosidically bound Neu5Gc in human brain, liver and blood plasma glycoproteins [67]. Glycoconjugate-bound Neu5Gc is the epitope recognized by the so-called Hanganutziu–Deicher (HD) antibodies. In various immunochemical studies the presence of these HD determinants has been shown in glycoproteins of various human healthy and cancer tissues and cell lines (for a detailed review see Ref. [8]). Even more convincing evidence for the presence of Neu5Gc in human tumors has been produced by GC–MS and ESI-MS [68–70]. Although classic studies tend to regard Neu5Gc as a tumor specific antigen, our study demonstrated no significant differences in Neu5Gc levels in serum of healthy women and women with endometrial cancer [11]. In view of the lack of CMP-Neu5Ac hydroxylase in human cells, the presence of Neu5Gc in human tissues might be attributed to the Neu5Gc-rich diet (normal component of most types of meat and fish) or endogenous alternative metabolic pathways, but these suggestions need further confirmation [7,8].

Normal total sialic acid level in serum/plasma ranges from 1.5 to 2.5 m*M* (0.5–0.8 g l^{-1}), with the free form only constituting 0.5–3 μM and the lipid associated forms 10–50 μM. The overwhelming

majority of serum sialic acids appear to be linked to glycoproteins (about 2 m*M*). No reliable differences between serum and plasma sialic acid concentrations have been reported. Sex does not appear to influence serum sialic acid levels. No variations in serum concentrations of total sialic acid were found in response to a meal [71]. Elevation of serum sialic acid concentrations has been related with pregnancy and aging [72,73]. Reports on the effect of aging are contradictory; Crook et al. support that the effect of aging is not dependent on sex, and female menopause is not related with serum sialic acid [73,74]. On the other hand, Ponnio et al. [75] reported that sialic acids do not increase with age in men but appear to increase with female menopause. In agreement with Crook et al. [74], determination of Neu5Ac in 12 pre- and 18 post-menopausal women by our research group showed no significant differences [11]. The effect of aging might be explained by the higher frequency of subclinically diseased individuals among the elderly subjects. Some studies have shown the positive association of smoking and elevated levels of sialic acids in men and that of serum sialic acid concentration and use of contraceptive pills in women [75,76]. The circulating concentrations of both C-reactive protein and total sialic acids remain unchanged following 1 year of smoking cessation [77]

In urine of healthy humans sialic acid is found in oligosaccharide compounds, bound to lactose and to *N*-acetyl-lactosamine. A considerably higher amount of free sialic acid in found in urine, typically 30–50% of the total sialic acids. The concentrations of about 40 μ*M* for free Neu5Ac and 250 μ*M* for bound Neu5Ac have been reported [78]. The presence of 2-deoxy-2,3-dehydro-Neu5Ac (Neu5Ac2en) in urine in a free form has been reported and the normal level is about 50 μ*M* [21]. Urinary free, bound and total sialic acids have been reported to be independent of sex and increase with age, while the relative proportion of free sialic acids changes only slightly over a lifetime. Certain diseases and pregnancy are characterized by both increased incidence and diversity of sialyloligosaccharides. Sialic acid storage disease is a rare autosomal recessive lysosomal storage disorder characterized by excessive urinary excretion of free sialic acid and accumulation of free sialic acid in skin fibroblasts. In sialuria, a rare defect with excessive synthesis of sialic acids, free sialic acid levels can be elevated 70- to 200-fold.

Human breast milk contains larger amounts of sialic acid (0.3–1.5 mg/ml) than bovine milk. Sialic acids are not present in the free form, but are bound in various glycoconjugates, such as oligosaccharides (about 75% of total sialic acid), glycolipids (mainly as monosialoganglioside 3 and disialoganglioside 3) and glycoproteins [79]. It is believed that the sialylated compounds in human milk serve to protect the infants against enteric infections [79].

4.2. Sialic acids in disorders and malignancies

Sialic acids rapidly increase in concentration after the onset of an inflammatory reaction or injury due to the fact that many acute phase proteins such as α_1-acid glycoprotein, α_1-antichymotrypsin and α_2-macroglobulin contain sialic acids. Significant correlation of serum concentration of total sialic acids with erythrocyte sedimentation rate, platelet count and neutrophil count was found. There was an inverse correlation with hemoglobin concentration and erythrocyte count [80]. Elevated serum sialic acids have also been reported in chronic liver diseases, pneumonia, rheumatoid arthritis, inherited disorders of sialic acids, alcoholism, Behcet's disease, Crohn's disease, renal diseases such as glomerulonephritis, chronic renal failure, diabetes mellitus with diabetic complications and atherosclerotic disease. There is a huge body of literature documenting concentrations of sialic acids in different disorders and it is beyond the scope of this article to review these data since it has been wonderfully done by other scientists [19,81]. The nonspecificity of determination of serum sialic acids is revealed and thus evaluation of the significance of sialic acids as diagnostic marker should be done very carefully. One research direction might be the search for specific altered glycosylation pattern in glycoproteins or lipoglycans; Ghosh et al. [82] suggest that the sialic acid-index of apolipoprotein J is a very sensitive marker for early detection of chronic ethanol consumption. In the following paragraphs, the relationship of elevated sialic acids with malignancies is more extensively presented.

Increased levels of serum total sialic acid con-

centrations have been reported in various patient groups suffering from brain tumors, leukemia, melanoma, malignant pleural effusion, carcinoma of the hypopharynx and larynx, cholangiocarcinoma, and cancers of the lung, ovary, endometrium, cervix, prostate, mouth, stomach, breast and colon (for a thorough review refer to Sillanaukee et al. [81]). The majority of these studies used colorimetric assays. Analysis with chromatographic and electromigration techniques, characterized with higher sensitivity and selectivity since other disturbances are avoided, confirms these results and shows that Neu5Ac is responsible for this increase [21,58]. In order to study this relationship, initial studies in our laboratories were performed in animal models. Serum and tissue specimens from healthy rats and rats with Walker 256 carcinoma as well as from healthy mice and mice with Lewis' lung cancer after metastasis were collected and analyzed for Neu5Ac and Neu5Gc by HPLC [14,15]. All mice tissues analyzed contained both types of sialic acids while Neu5Gc was the major one (>99%). Tissue samples from mice with metastatic cancer showed a significant increase (67–200%) of total sialic acids mainly as a result of increased synthesis of the predominant sialic acid type, Neu5Gc. Accordingly, total sialic acid content in serum from mice with cancer was four times higher than in healthy animals, with no changes in the Neu5Ac–Neu5Gc molar ratio, indicating a similar rate of Neu5Gc and Neu5Ac synthesis [14]. In rats, both neuraminic acids were identified, but Neu5Ac was the predominant sialic acid. Samples from rats with generalized metastasis showed a significant increase (45–80%) of total sialic acids. A 1.8 times increase of total sialic acids in serum of rats with cancer and no differences in the Neu5Ac–Neu5Gc ratio were recorded [15]. In both animal models increased synthesis of predominant sialic acids was revealed.

Study of Neu5Ac and Neu5Gc in serum of women before and after surgical treatment of early endometrial cancer and of the relation of their levels with the progress of surgical therapy produced similar results [11]. In patients with endometrial cancer the total sialic acid level before surgical treatment was significantly (3.3 times) higher than that of the control group, suggesting oversialylation of serum proteins. The elevation in sialic acid level was exclusively due to Neu5Ac [11]. In general, sialic acids, being the outermost termini of glycoproteins and glycolipid oligosaccharide side-chains, undergo substantial variations during malignant transformations since carcinogenesis is related with changes in cell membrane and organelle components, which in turn cause deregulation of cell growth and proliferation. Several different mechanisms are assumed to underlie the elevated sialic acid concentrations in various cancers. Increased activity of sialyltransferases, leading to an increased amount of sialic acid on the cell surface and the spontaneous release or shedding of aberrant sialic acid-containing cell surface glycoconjugates [83], may cause elevation of sialic acid concentration in plasma. Alternatively, increased activity of serum or tissue sialidase [84] in conjunction with the inflammatory response (increase of concentrations of acute phase proteins) could elevate serum sialic acid levels [81].

The use of sialic acid levels as prognostic and diagnostic indicator for several malignancies has been investigated. The majority of the clinical studies reveal that the clinical utility of sialic acid determination for screening cancer patients is limited because of its apparent nonspecificity to a given disease [81,85]. In addition, several non-pathological factors, such as aging, pregnancy, use of contraceptive pills and smoking may cause changes in sialic acid concentrations. We thereby believe that determination of serum sialic acid content is not appropriate for differential cancer diagnosis or for early detection of cancer.

In our study of Neu5Ac and Neu5Gc in serum of women before and after surgical treatment of early endometrial cancer it was demonstrated that serum Neu5Ac levels significantly decreased after surgical therapy [11]. In one case, Neu5Ac level increased 15 days and 8 months after surgery (1.8 and 2.5 times as compared to control, respectively), and a metastasis not detected during surgery was recorded. The obtained results suggest that Neu5Ac level in serum may be used as a tumor marker in evaluating the suitability of surgical treatment in early endometrial cancer. Several reports have also described normalization of sialic acid levels after the successful treatment of cancer, there being subsequent elevation of sialic acid levels with recurrent or metastatic malignancy [81,86,87]. We therefore suggest that

measuring sialic acid concentration cannot provide information as a stand-alone marker but in combination with other markers may be useful in disease screening, progression follow up and in monitoring response to treatment.

Analysis of *O*-acetylated sialic acids, mostly neglected so far, might reveal more reliable and specific diagnostic markers and even new therapeutic routes. Recent studies using specific lectins and antibodies have demonstrated specific altered *O*-acetylation patterns in malignancies and identified them as oncofetal antigens [17,18]. For example, colorectal cancer, ulcerative colitis and colonic adenoma have been associated with decreased 9-*O*-acetylation, but increased sialylation of mucin and neo-expression of 4-*O*-acetyl-sialic acids [88–90]. Application of the recent advances in LC–MS techniques to study of *O*-acetylation patterns in human tissues is necessary for elucidation of the *O*-acetylation processes since it provides indisputable reliable data for trace amounts of biologic samples and it can determine the whole *O*-acetylation pattern in contrast to traditional immunochemical techniques.

4.2.1. Nomenclature

Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid;
KDN	2-keto-3-deoxy-nonulosonic acid
Me	methyl
Ac	acetyl
Lt	lactoyl
S	sulfate
CE	capillary electrophoresis
LPS	lipopolysaccharide
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
GC	gas chromatography
LC	liquid chromatography
ESI	electrospray ionization
HPAEC–PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
Tos-Cl	*p*-toluenesulfonylchloride
DMB	1,2-diamino-4,5-methylene-dioxybenzene
NPNA	*N*-propionylneuraminic acid
MALDI–TOF–MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
CZE	capillary zone electrophoresis
EM	electrophoretic mobility
EOF	electroosmotic flow
AMAC	aminoacridone
APTS	8-aminopyrene-1,3,6-trisulfonic acid
ANDSA	7-aminonaphthalene-1,3-disulfonic acid
NBD	7-nitro-2,1,3-benzoxadiole
RSD	relative standard deviation
HD	Hanganutziu–Deicher
Neu5Ac2en	2-deoxy-2,3-dehydro-Neu5Ac
PMP	1-phenyl-3-methyl-2-pyrazolin-5-one
LIF	laser-induced fluorescence

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