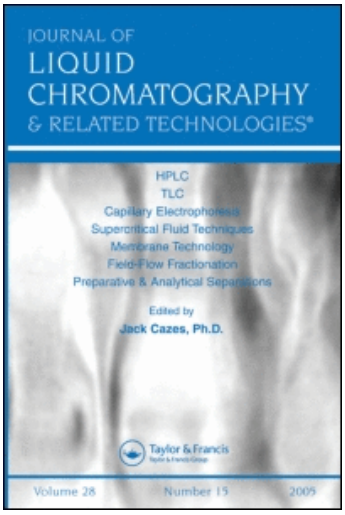

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A METHOD TO QUANTITATE TOTAL SIALIC ACID, GLUCOSAMINE, AND GALACTOSAMINE IN BLOOD SERUM AND GLYCOCONJUGATES BY HPLC

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ABSTRACT

A sensitive method for determining the total amount of sialic acid, glucosamine, and galactosamine in blood serum and glycoconjugates using reversed phase high performance liquid chromatography is described. Sialic acids present in glycoconjugates are released by acidic hydrolysis with 2 M TFA at 80°C for 2 h, whereas hexosamines are released following hydrolysis with 6 M HCl at 100°C for 8 h. The sialic acids released under these conditions are identified as a decarboxylated and deacylated product bearing a primary amino group (amino-

product). Following removal of neutral monosaccharides and amino acids by ion-exchange chromatography on Dowex 50x8, the obtained amino-product and hexosamines are converted to Tosyl-derivatives. The obtained derivatives of amino-product, glucosamine, and galactosamine, are completely separated and determined on a Supelcosil LC-18 column by means of isocratic elution using a mobile phase of water-acetonitrile (85:15, v/v) and detection at 231 nm. 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 highly accurate when compared with literature values. The present method has the advantage of permitting determination of total sialic acid, glucosamine, and galactosamine in glycoconjugates and blood serum using the same HPLC conditions.

INTRODUCTION

Sialic acids (SA) is a trivial name used to describe a family of neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosic acid) derivatives. They are found as constituents of glycoconjugates in viruses, bacteria, protozoa, as well as in cells and tissues of a wide range of higher animals. The most frequently encountered sialic acids are the two N-substituted forms: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). These molecules are often acetylated on the hydroxyl groups of C- 4, 7, 8 and 9, forming over 30 isomers.[1, 2]

Sialic acids are also constituents of glycoproteins and/or glycolipids, most often located at the non-reducing terminal position of the oligosaccharide side chains. They are linked by glycosidic bonds to other monosaccharides usually involving galactose and N-acetyl galactosamine. Furthermore, sialic acid polymers with an oligo- and/or polysialyl sequence have been demonstrated as components of certain glycoproteins and gangliosides.[3]

Elevated levels of sialic acids are found in serum of patients suffering from cancer, congenital metabolic disorders, and various other diseases[4-8] and, therefore, have been proposed as a potential tumor marker. The bulk of serum sialic acids is bound to glycoproteins or glycolipids.

There are two basic procedures for liberating SA from glycosidic linkages; enzymic treatment, using sialidases which differ in specificity for sialic acid glycosidic linkages and the sialic acid type,[9,10] and acidic hydrolysis, using a variety of inorganic and organic acids.[1-15] With most acids the liberation of SA from the oligosaccharide chains is followed by simultaneous removal of O-acetyl groups, whereas the N-acyl, i.e. N-acetyl and N-glycolyl linkages, are

usually resistant. Most sialic acids can, thus, be recovered in their two basic forms, Neu5Ac and Neu5Gc. The disadvantage of using hydrolytic procedures is that almost always there is association with some destruction of the carbohydrates. The extent of degradation, however, may be reduced by using very mild acidic conditions such as 25-100 mM HCl or trifluoroacetic acid (TFA) as we have previously described.[16]

A number of methods such as spectrophotometric determinations,[12, 13, 17-21] enzymic digestions,[22] thin-layer chromatography,[13] gas chromatography,[13, 23-25] gas-liquid chromatography combined with mass spectrometry,[26-28] ^{1}NMR spectrometry,[29, 30] and high performance liquid chromatography (HPLC) are used for measuring sialic acids. Cation-[31] and anion-exchange resins,[9-12, 26, 32-36] as well as, reversed-phase resins[3,16,37] have been used in various HPLC procedures. The sensitivity of HPLC methods is dependent on the detection mode used and the performance may be improved with postcolumn reactions[38] or suitable precolumn derivatization. Detection limits reported vary from a few nanograms to some picograms. Recently, a number of capillary electrophoretic methods for sialic acid determination have been developed.[39]

In spite of the huge variety of methods available, analysis of sialic acids in biologic samples remains a difficult task. Pilot experiments in our laboratories showed that hydrolyzing glycoconjugates in strongly acidic conditions, a mixture containing glucosamine (GlcN), galactosamine (GalN), and an amino-product is obtained. Similarly, treatment of Neu5Ac and/or Neu5Gc standards with 2 M TFA at 80°C for 2 h, conditions required for complete liberation of sialic acids, leads to formation of this amino-product suggesting that it is coming from sialic acid hydrolysis. The aim of this investigation was, therefore, to determine the total sialic acid content in glycoconjugates by analyzing this amino-product instead of the various forms of sialic acids. Modifying a suitable and successful HPLC method previously used for analysis of hexosamines in glycosaminoglycans,[40] we describe a precise method able to determine the amino-product derived by the acidic hydrolysis of sialic acids, as well as, glucosamine and galactosamine, under the same chromatographic conditions.

EXPERIMENTAL

Materials

Neu5Ac, Neu5Gc, N-acetylneuraminyl-α-(2,3)-lactose from human milk, GlcN hydrochloride, GalN hydrochloride, fetuin (type III) from foetal calf serum, and mucin (type I-S) from bovine submaxillary glands were purchased

from Sigma (St. Louis, MO, USA). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, USA). All other chemicals used were of analytical grade.

p-Toluenesulfonylchloride or Tosylchloride (Tos-Cl) reagent was prepared by dissolving 50 mg Tos-Cl in 10 mL of acetone. Triethylamine reagent was prepared by diluting 100 μL of triethylamine in 10 mL of acetone. Solutions were stored at 4^0C in dark and were found to be stable for one week.

Instrumentation, HPLC Conditions and Quantitation

An LDC constametric III pump equipped with a Reodyne Model 7125 injector unit with a 50 μL loop and an LDC spectromonitor 1204 A UV detector with 8 μL flow cell was used. Separation was performed on a Supelcosil LC-18 (250 mm x 4.6 mm i.d., particle size 5 μm) column (Supelco, Bellfonte, PA, USA), equipped with a RP-18 (30 mm x 4.6 mm i.d.) precolumn (Brownlee Labs., Santa Clara, CA, USA). Samples were chromatographed using 15 % (v/v) aqueous acetonitrile at ambient temperature and a flow rate of 1.0 mL/min. Eluted peaks were recorded at 231 nm. Quantitation was performed by comparing the peak areas obtained from samples with those obtained using standard solutions. Calibration curves were constructed by injecting known amounts of standard solutions of GlcN, GalN, Neu5Ac, and Neu5Gc treated under the same acidic conditions used for sample analysis. Analysis of standard solutions was performed by serial dilutions of 0.1 and 5 mM stock solutions.

Sample Treatment and Preparation of Standards

The protocol followed for the analysis of sialic acids and hexosamines in glycoproteins is presented in Figure 1. Glycoconjugates containing 0.5-5 μg sialic acids were hydrolyzed in 500 μL of 2 M TFA at 80^0C for 2 h in screw-capped polypropylene microtubes. Sialic acid standards were prepared by treating known amounts of Neu5Ac and/or Neu5Gc under the same conditions. For the analysis of total sialic acid content in blood serum, 10 μL were hydrolyzed in 500 μL of 2 M TFA at 80°C for 2 h and then chromatographed as described below. Samples with 0.5-5 μg hexosamine content were hydrolyzed in 500 μL 6 M HCl at 100°C for 8 h.[41] Hydrolysates of both cases were lyophilized and the obtained residues were dissolved in 200 μL of 2 x distilled water. Following chromatography on a Dowex 50X 8 column (H^+ form, 40 mm x 3 mm i.d.) and subsequent washing with 1 mL of 2 x distilled water and 1 mL of 0.1 M HCl, amino sugars were eluted with 1 mL of 2 M HCl. The later fraction was collected and lyophilized.

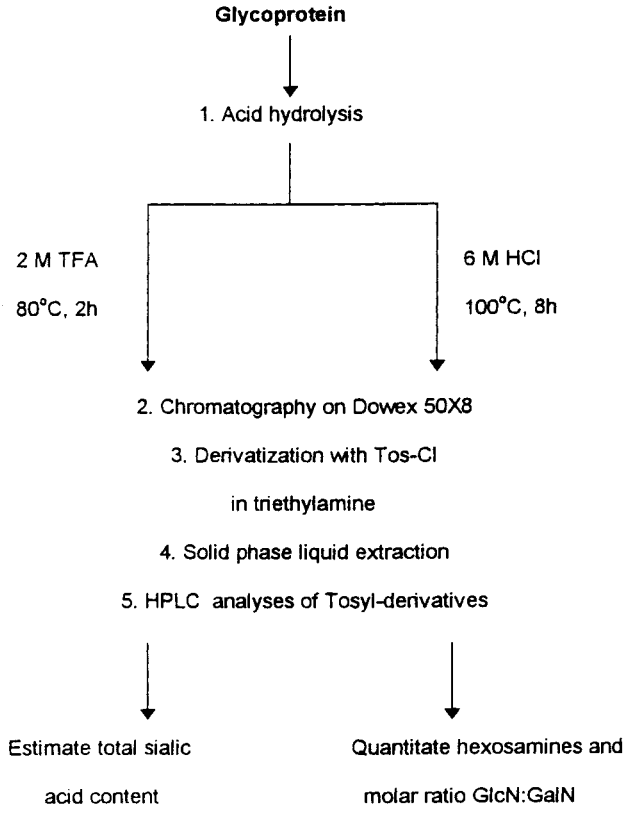

Figure 1. Protocol applied for the determination of total sialic acid content, glucosamine, and galactosamine in glycoproteins. The entire method requires two different hydrolytic procedures and HPLC analyses, using the same HPLC separation conditions and detection mode.

Derivatization Procedure

Tosyl-derivatives were prepared by a modification of the procedure reported by Hjerpe et al.[40] In particular, 25 μL of water, 37.5 μL of Tos-Cl reagent and 37.5 μL of triethylamine solution were sequentially added to dry hydrolysates and the mixture was heated at 60°C for 40 min. The reaction mixture was dried and then dissolved in 100 μL of methanol and 800 μL of water. Excess of reagents and non-tosylated derivatives were removed by passing the mixture through a Sep-Pak C_{18} cartridge, which had been equilibrated with 5 mL of methanol and 10 mL of water. The cartridge was

washed with 1.5 mL of 10% (v/v) methanol and Tosyl-derivatives were eluted with 3 mL of 50% (v/v) methanol. Following evaporation of the latter fraction, the residue was dissolved in 200 μL of acetonitrile and aliquots of 10-20 μL were injected into the column. When larger volumes had to be injected, the dried derivatives were preferably dissolved in 15% (v/v) aqueous acetonitrile.

RESULTS AND DISCUSSION

Hydrolytic Release of Sialic Acids and Hexosamines from Glycoconjugates

In order to separate and determine sialic acids and hexosamines it is necessary to liberate them from the glycoconjugates (glycoproteins, glycolipids and glycosaminoglycans). Although hydrolysis with 2 M TFA at 80°C for 2 h does not ensure strong acidic conditions able to liberate hexosamines, sialic acids are almost completely released with a simultaneous removal of their acyl groups. The liberated sialic acids do not exhibit any ability to bind on the anion exchanger Dowex 1X8 ($-N^+R_3$ active group) indicating that the sialic acids are decarboxylated. However, this product is retarded on the cation exchanger Dowex 50X8 ($-SO_3^-$ active group). Since this ability is lost following treatment with low-pH nitrous acid,[42] the presence of a primary amino group in the deacylated and decarboxylated product, generally named amino-product, is concluded.

Treating various amounts of Neu5Ac and Neu5Gc in this way, it was found that the amount of the resulting amino-product is proportional to the amount of SA used for hydrolysis. This suggests that the quantitative analysis of sialic acid content in biologic samples can be achieved only when standard solutions are simultaneously treated under the same conditions. Possible interference due to amino acids present in hydrolysates is eliminated by ion-exchange chromatography according to Boas procedure.[43]

The effect of the hydrolysis time on the liberation of SA with 2 M TFA at 80°C was studied using fetuin and mucin as substrates. As shown in Figure 2, SA are liberated within 2 h, whereafter there is some destruction depending on the time of hydrolysis. Therefore, hydrolysis with 2 M TFA for 2 h was selected for liberation of SA present in glycoproteins. The amount of hexosamines liberated from glycoproteins with this procedure is very low (<10%). Hexosamines were, therefore, liberated following a hydrolysis step with 6 M HCl at 100°C for 8 h.[41] The latter conditions are not suitable for sialic acids release, since a significant destruction (> 90%) is recorded.

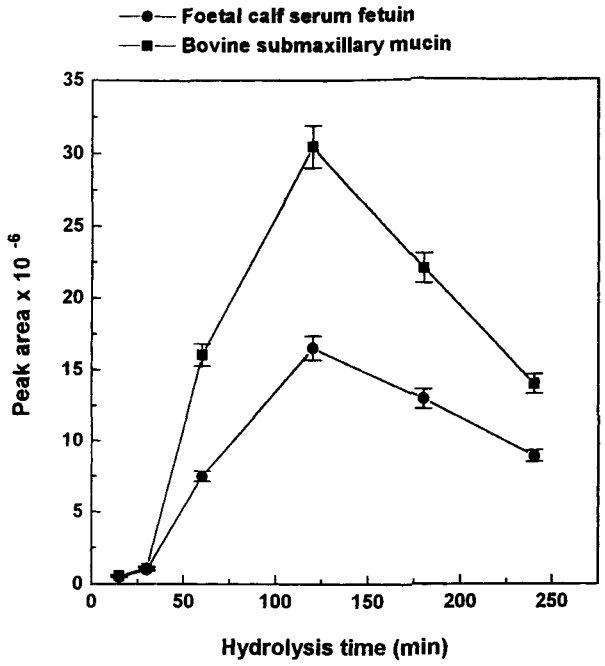

Figure 2. Rate of liberation of sialic acids from foetal calf serum fetuin (●) and bovine submaxillary mucin (■) using hydrolysis with 2 M TFA at 80°C. Results are expressed as released amino-product versus time of hydrolysis. Complete release is achieved after 2 h of hydrolysis. Given values represent the average ± s.d. of three experiments in triplicate.

Derivatization and Separation of Tos-aminosugars

Sulphonyl chlorides have been used previously to detect primary amino groups in proteins[44] and hexosamines.[45] According to Hjerpe et al.,[40] tosylation of amino sugars is performed in an aqueous system containing water-miscible organic solvent and inorganic base. In the present modified procedure, the reaction time is improved by using triethylamine instead of inorganic base. Moreover, triethylamine is easily removable upon lyophilization.

Under these conditions, it was found that radioactively labeled glucosamine is converted to Tosyl-derivatives with a recovery exceeding 94%, suggesting a high yield of derivatization.

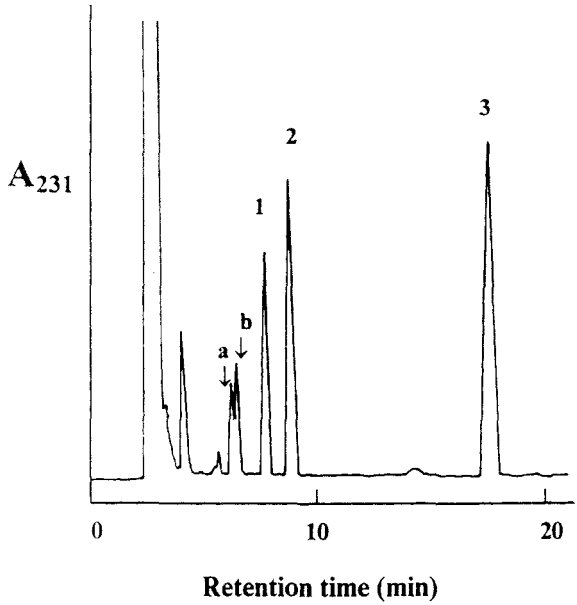

Figure 3. Typical chromatogram of Tosyl-derivatives of GalN (peak 1), GlcN (peak 2) and the sialic acid-derived amino-product (peak 3) prepared by treating of Neu5Ac and/or Neu5Gc with 2 M TFA at 80°C for 2 h. Arrows *a* and *b* indicate the anomeric forms of GalN and GlcN, respectively. Chromatography was performed on a reversed phase Supelcosil LC-18 column eluted with 15% (v/v) aqueous acetonitrile at 1.0 mL/min. Eluted peaks were recorded at 231 nm.

Reversed-phase HPLC separation of Tosyl-derivatives of GlcN and GalN has been successfully carried out by Hjerpe et al. [40] on a Hypersil ODS column with 8% (v/v) aqueous acetonitrile containing 0.1% (w/v) Brij-35. In order to separate Tosyl-derivatives of sialic acids and hexosamines, a reversed-phase Supelcosil LC-18 column eluted with 15% (v/v) aqueous acetonitrile was used. As shown in Figure 3, each one of N-Tosyl-galactosamine and N-Tosyl-glucosamine results in two peaks with retention times 6.30 and 7.75 min for the former (arrow a and peak 1, respectively) and 6.55 and 8.90 min (arrow b and peak 2, respectively) for the later.

These double peaks for each hexosamine represent most probably the β and α anomeric forms. Tosyl-derivatives of Neu5Ac and Neu5Gc, treated separately with 2 M TFA at 80°C for 2 h, result in one peak at 17.40 min (Figure 3, peak 3).

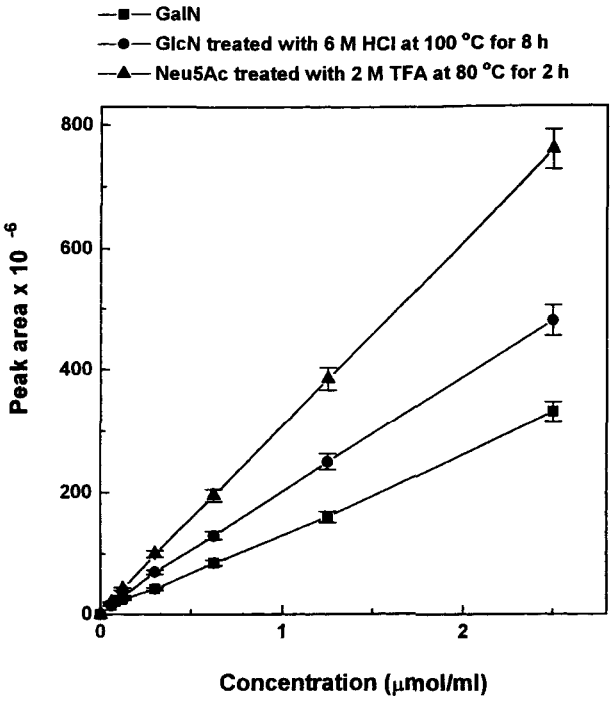

Figure 4. Calibration graphs obtained by plotting peak areas versus the concentrations of GalN (■) and GlcN (●) treated with 6 M HCl at 100°C for 8 h and of Neu5Ac and/or Neu5Gc (▲) treated with 2 M TFA at 80°C for 2 h. Results are expressed as the average of three experiments in triplicate. The detector response corresponds to the following equations: $y = (-0.623 + 14.415\ x)\ 10^7$ ($sd = 1.234\ 10^7$) for GalN, $y = (1.733 + 19.392\ x)\ 10^7$ ($sd = 3.67\ 10^7$) for GlcN and $y = (1.891 + 30.099\ x)\ 10^7$ ($sd = 1.822\ 10^7$) for SA-derived amino-product.

Sensitivity, Linearity and Accuracy

The sensitivity and linearity of the method were tested by using Neu5Ac and/or Neu5Gc, hydrolyzed with 2 M TFA at 80°C for 2 h, as standards for sialic acid analysis. GlcN.HCl and GalN.HCl, hydrolyzed with 6 M HCl at 100°C for 8 h, were used as standards for hexosamine analysis. As shown in Figure 4, the detector response of the Tosyl-derivatives is linear within the range of 0.125 - 25 nmol of injected compound, indicating a high linearity range. The precision of the method was determined by twelve repeated determinations using all aminosugars in a mixture.

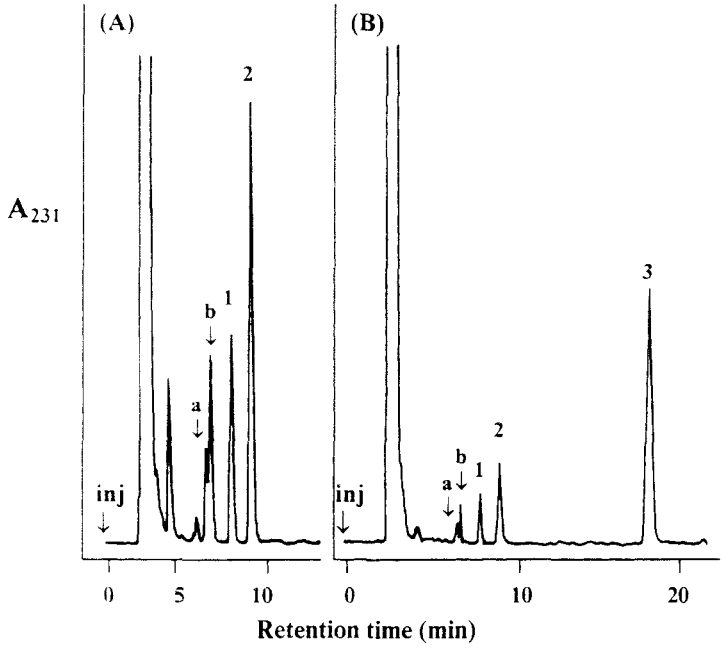

Figure 5. Application of the HPLC method for the analysis of hexosamines and sialic acids in bovine submaxillary mucin hydrolysates. Analysis for GalN and GlcN was performed following hydrolysis of glycoproteins with 6 M HCl at 100°C for 8 h (A) and for total sialic acid with 2 M TFA at 80° C for 2 h (B). Column, eluant, and conditions as in Fig. 3.

When 2.5 nmol are used, the relative standard deviation is 2.8% for glucosamine, 2.6% for galactosamine, and 3.0% for sialic acid, indicating a precise method. The detection limit, expressed as twice the baseline noise, corresponds to 10 pmol of glucosamine, 12 pmol of galactosamine, and 6 pmol of sialic acid injected, indicating a highly sensitive method.

The peak height and the peak area ratios using equivalent amounts of glucosamine, galactosamine, and sialic acids (2.5 nmol) are 1.37:1:1.55 and 1.36:1:2.15 , respectively.

The minimum required amount of glycoprotein (containing 5% SA) for accurate determination of sialic acids and hexosamines with a confidence interval of 95%, is 5-10 μg dry weight.

Table 1

Total Sialic Acid, Glucosamine, and Galactosamine Content in Two Glycoproteins and Blood Serum as Determined by HPLC[a]

Sample	SA[b]	GalN[c]	GlcN[c]	Ref.
Bovine sub-maxillary mucine type I-S	20.8±1.3 (20.9-22)[d]	9.6±0.4 (14.8-25.2)[f]	14.4±0.8	12, 13, 16
Fetuin type III	7.9 ±0.3 (7.8-8.7)	0.4±0.02 (≤ 1.1)	4.8±0.03 (5.6)	46, 47
Human Serum	269-527[e] (309-896)	---	---	48

[a] SA and hexosamines were determined by the same HPLC method using different hydrolytic conditions for their release from glycoproteins. Results are expressed as percent of the glycoproteins dry weight.
[b] SA content was determined comparing the peak areas of samples with those of the amino-product derived by hydrolysis of standard Neu5Ac.
[c] GalN and GlcN contents were determined following hydrolysis with 6M HCl at 100°C for 8 hr.
[d] Values in parenthesis show the percentage given in literature.
[e] Content of sialic acid in blood serum is expressed in μg/mL.
[f] Values in parenthesis represent total hexosamine content.

Applications

The liberation of sialic acids present in the non-reducing terminal of glycoconjugates is most often achieved by mild acidic hydrolysis with 0.1 M HCl or 25 mM TFA.[11-16] Choosing a stronger acidic hydrolysis with 2 M TFA at 80°C for 2 h, however, a complete transformation of both Neu5Ac and Neu5Gc to the same amino-product is obtained and this compound may be analyzed by a convenient reversed-phase HPLC method. As shown in Figure 5 and summarized in Table 1, SA content in glycoproteins determined by the present method is in close agreement with those reported earlier,[12, 13, 16, 46, 47] indicating a highly accurate method.

The various glycoconjugates present in human serum contain sialic acids. Application of the proposed HPLC method for analyzing SA in human serum of six healthy individuals showed that the total sialic acid content is in the

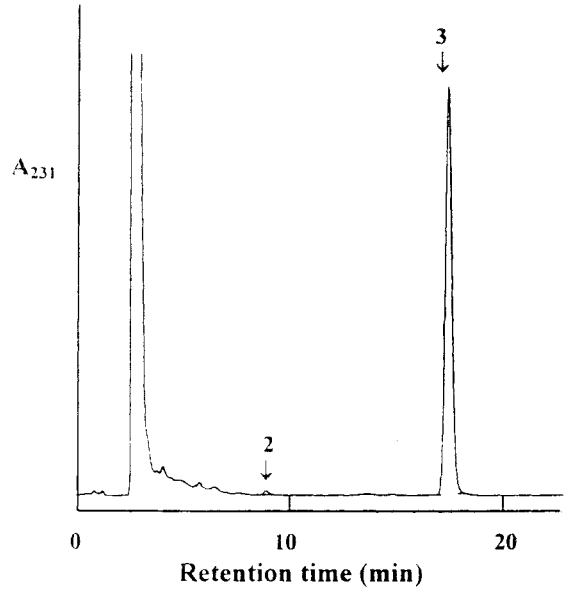

Figure 6. Chromatogram showing the analysis of total sialic acid in blood serum. Hydrolysis of sample was performed with 2 M TFA at 80°C for 2 h. Column, eluant, and conditions as in Fig. 3.

range of 269 - 527 μg/mL (Figure 6 and Table 1). A higher level of total sialic acids in human serum was estimated by chromatometric methods[48] (309 - 896 μg/mL). This finding is probably due to the fact that most of chromatometric methods were developed as modifications of assays previously used for measurement of other carbohydrates, and therefore, false elevated amounts are obtained when they used for the analysis of biologic samples.

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