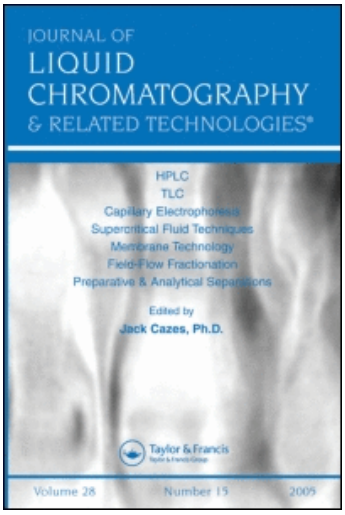

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SAMPLE CLEAN-UP AND ANALYSIS OF N-ACETYL AND N-GLYCOLYLNEURAMINIC ACIDS IN BLOOD SERUM AND TISSUE SPECIMEN BY HPLC

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ABSTRACT

A procedure for sample clean-up, isolation of glycoconjugates, and analysis of the two major sialic acids, Neu5Ac and Neu5Gc, as per-O-benzoylated derivatives in biologic samples is described. In blood sera, the bulk of low molecular weight molecules was efficiently removed by precipitation of sialic acids containing glycoconjugates with saturated ammonium sulphate, pH 10, at 0°C, centrifugation, and gel chromatography on a Sephadex G-25. The tissue specimen was homogenized with water at 0°C and following centrifugation the water-soluble sialic acids containing glycoconjugates were recovered in the supernatant. Sialic acids were liberated from their isolated macromolecules by acidic hydrolysis with 25 mM trifluoroacetic acid at 80°C for 2 h. Constituents with positive charged groups and neutral monosaccharides were removed by ion-exchange chromatographies on Dowex 50X8 and Dowex 1X8, whereas sialic acids were eluted with 2M formic acid.

Following derivatization with benzoic anhydride in the presence of *p*-dimethylaminopyridine, the per-O-benzoylated derivatives were completely separated and analyzed on a Supelcosil LC-18 column by isocratic elution with acetonitrile-water (67:33, v/v) and detection at 231 nm. Quantitation was performed using peak areas obtained from external standards treated under the same hydrolytic conditions as samples. Application of the method in C57BI mouse lung showed that N-glycolylneuraminic acid is the major sialic acid and that the amounts of sialic acids in rat serum are in excellent agreement with those previously reported.

INTRODUCTION

N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) are the most commonly occurring derivatives of a family of compounds with the trivial name sialic acids (SA) derived from neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosic acid), which is often acetylated on the hydroxyl groups of C-4, 7, 8, and 9.[1,2] They are constituents of glycoconjugates in microbes, protozoa, as well as in cells and tissues of higher animals located usually on the outer cell membrane. There have been reported increased serum levels of sialic acids in patients suffering from cancer,[3] myocardial infarction,[4,5] and various other diseases.[6-10] In addition, Neu5Gc was identified in human and chicken cancerous tissues.[11-13]

A number of methods are available for the measurement of total sialic acid content but very few have been reported for the determination of Neu5Ac and Neu5Gc.[13-19] Sialic acids are liberated from glycoconjugates either by a variety of sialidases which differ in specificity for sialic acid type and linkage,[14,20] or by acidic hydrolysis,[21,22] where the liberation is followed by simultaneous removal of O-acetyl groups. The majority of sialic acids can thus be converted in their two basic forms, Neu5Ac and Neu5Gc. The liberation of SA by acidic hydrolysis has the disadvantage of some destruction, which may, however, be reduced in very mild acidic conditions.[15] In spite of the variety of analytical methods for sialic acids available, analysis of Neu5Ac and Neu5Gc in biologic samples remains a difficult task. Applying a sensitive HPLC method reported by Karamanos et. al.[15] and following the proposed sample clean-up procedure, analysis of Neu5Ac and Neu5Gc in biologic samples has been successfully accomplished. This analytical strategy opens a new area in studying the relationship of Neu5Ac and Neu5Gc synthesis and functions in blood sera and tissues of healthy individuals and patients suffering from cancer.

MATERIALS AND METHODS

Materials

Rats and male C57BI mice were obtained from the Experimental Laboratory of Theagenian Cancer Institute (Thessaloniki, Greece). Neu5Ac, Neu5Gc and N-acetylneuraminyl-*a*-(2,3)-lactose from human milk 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.

Benzoylation mixture was prepared by dissolving 1 g of benzoic anhydride and 0.5 g of *p*-dimethylaminopyridine in 10 mL of pyridine. The solution was stored at 4°C and was stable for more than a week.

Sample Treatment and Preparation of Standards

Tissue

Mice were sacrificed after cardiac exsanguination under ether anesthesia, lungs were collected, weighed, and homogenized at 0°C for 1 min with 3 mL of water following washing of the homogenizing system with 1 mL of water. The obtained fluid was centrifuged at 11,000 x *g* for 5 min, the supernatant was collected and kept at -40°C for SA analysis.

Blood serum

To 100 μL of serum 2 volumes of saturated ammonium sulphate, pH 10, were added and the mixture was kept at 0°C for 20 min. Following centrifugation at 11,000 x *g* for 5 min, the obtained precipitate was dissolved in water (200 μL) and the solution was chromatographed on a Sephadex G-25 (30 mm x 13 mm i.d.) prepacked column (PD-10, Pharmacia, Sweden). The column was washed with 0.8 mL of water, the sialic acid containing macromolecules were collected by elution with 1.5 mL of water and kept at -40°C for further analysis.

Hydrolysis and purification

Glycoconjugates containing 5-50 μg total sialic acids were hydrolyzed in 500 μL of 25 mM trifluoroacetic acid (TFA) at 80°C for 2 h in screw-capped polypropylene microtubes. Standards were prepared by treating known

amounts of Neu5Ac and Neu5Gc under the same conditions. Hydrolyzates were lyophilized and the obtained residues were dissolved in 200 μL of 2 x distilled water. The solutions were chromatographed on a Dowex 50X8 (40 mm x 3 mm i.d.) column washed with 1 mL of water. The elutes were partly neutralized to a pH 3-5 with 0.5 M NH_4OH and then were rechromatographed on a Dowex 1X8 (200-400 mesh, $HCOO^-$ form, 40 mm x 3 mm i.d.) column.[23] The column was washed with 1 mL of water and sialic acids were eluted with 2 mL of 2 M HCOOH. The later fraction was collected, lyophilized and taken for benzoylation.

Derivatization Procedure

Per-O-benzoylated derivatives were prepared by a previously described procedure.[15] In particular, 100 μL of benzoylation mixture were added to the dry hydrolyzates and the mixture was heated at 80°C for 20 min. The remaining amount of benzoic anhydride was destroyed by adding 0.9 mL of water and heating the mixture for a further 10 min at 80°C. Excess of reagents and under-benzoylated 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.

After sample addition to the cartridge, it was washed with 5 mL of water and the per-O-benzoylated derivatives of sialic acids were eluted with 5 mL of acetonitrile. Following evaporation of the later fraction, the residue was dissolved in 200 μL of acetonitrile and aliquots of 10-20 μL were injected into the column.

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 column (250 mm x 4.6 mm i.d.), particle size 5 μm (Supelco, Bellfonte, PA, USA), equipped with a RP-18 pre-column (30 mm x 4.6 mm i.d., Brownlee Labs., Santa Clara, CA, USA).

The samples were chromatographed with 67 % (v/v) aqueous acetonitrile at ambient temperature and at a flow rate of 1.5 mL/min. The eluted peaks were recorded at 231nm. Quantitation was performed by comparing the peak areas obtained from the samples with those obtained using standard solutions.

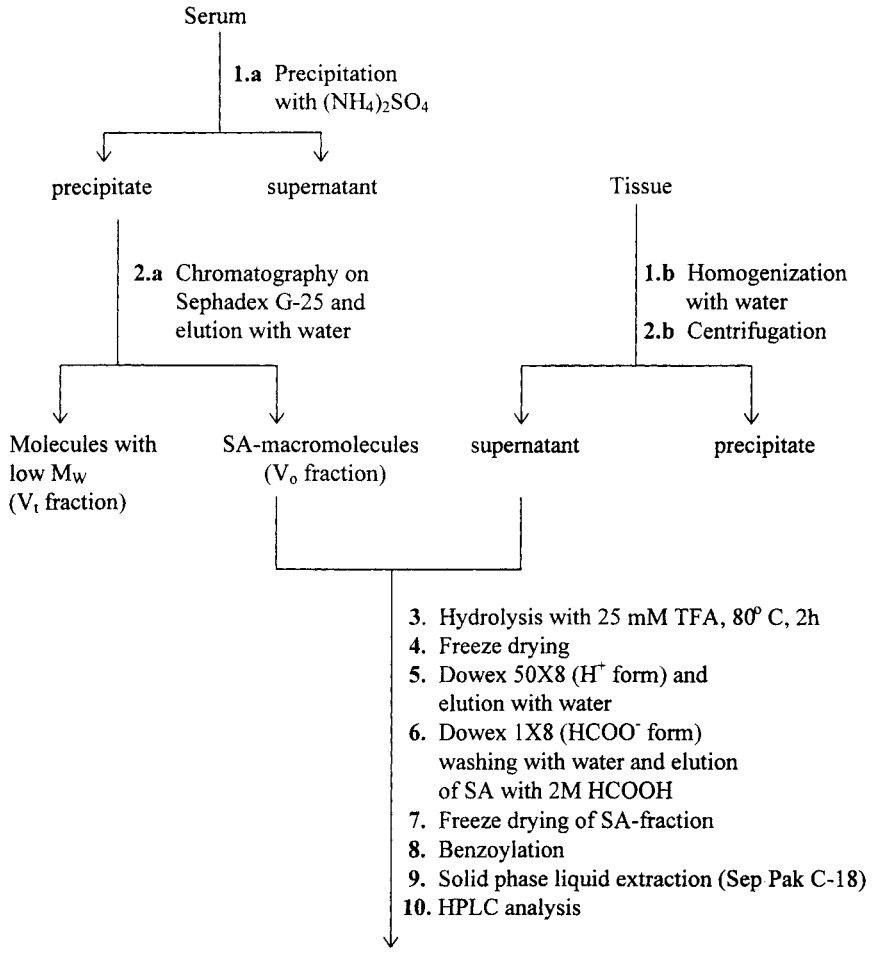

Figure 1. Flow chart applied for the isolation of sialic acids containing glycoconjugates and determination of Neu5Ac and Neu5Gc in blood serum and tissue specimen.

RESULTS AND DISCUSSION

In order to separate and determine sialic acids it is necessary to liberate them from the glycoconjugates (glycoproteins, glycolipids, and glycosaminoglycans). Hydrolysis with 25 mM HCl or TFA for 2 h at 80°C

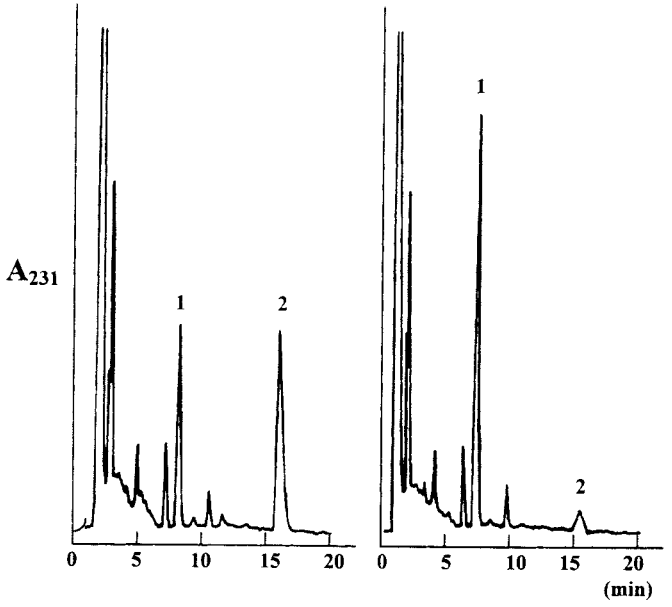

Figure 2. (A), typical chromatogram of per-O-benzoylated derivatives of Neu5Ac (peak 1) and Neu5Gc (peak 2) and (B), application of the method for the analysis of SA in rat serum. Chromatography was performed on a reversed phase Supelcosil LC-18 column eluted with 67% (v/v) aqueous acetonitrile at 1.5 mL/min. Eluted peaks were recorded at 231 nm.

seems to be the best of the tested alternative procedures described in literature.[15] The schematic diagram used for hydrolysis and analysis of sialic acids in biologic samples is presented in Figure 1. The possibility that some O-acetyl groups are not removed after this hydrolysis, especially in biologic samples, should be taken into account. Such derivatives would then elute in a reversed phase HPLC earlier than the corresponding per-O-benzoylated derivatives. In preparations where the possibility of remaining O-acetyl groups still must be considered, this problem may well be overcome by a complementary alkaline hydrolysis with 4 M NH_4OH at 25°C for 4 h, as it has been previously proposed.[15,23]

Benzoylation of Neu5Ac and Neu5Gc was performed by adding benzoic anhydride in the presence of *p*-dimethylamino-pyridine in dry and fresh distilled pyridine.[15] Per-O-benzoylated derivatives of both sialic acids were

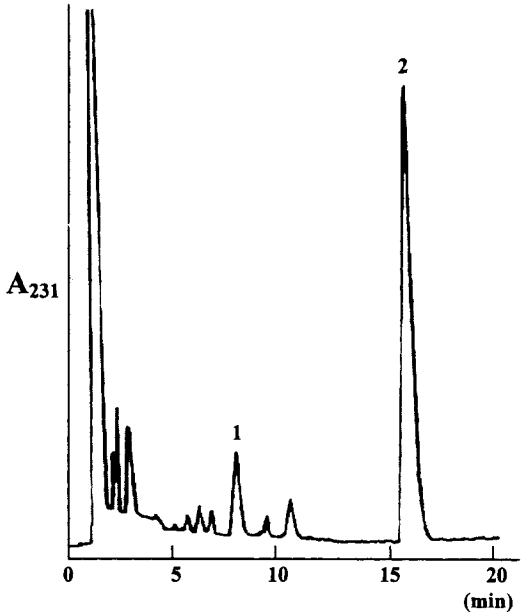

Figure 3. Chromatogram obtained by analysis of SA in tissue (C57BI mouse lung) specimen. Column, eluant, and conditions as in Fig. 2.

completely separated on a reversed-phase Supelcosil LC-18 column eluted with 67% (v/v) aqueous acetonitrile (Fig. 2A). The retention times for Neu5Ac and Neu5Gc were 8.4 ± 0.4 and 16.8 ± 0.8 min, respectively. To avoid interference of neutral hexoses and hexosamines, which have retention times between 9 and 16 min, the dried hydrolysates were subjected on Dowex 50X8 (H^+) and Dowex 1X8 ($HCOO^-$) columns before derivatization.

The low molecular weight (M_W) molecules of serum, interfering with the analysis, have been successfully removed by precipitation of serum macromolecules with saturated ammonium sulphate at pH 10 and subsequent gel chromatography on a Sephadex G-25 column (PD-10), where the void volume fraction was collected. Analysis of this fraction for sialic acids showed that approximately 98.5% of total SA were recovered. The method, as it has been previously reported,[15] gave linear response in the interval tested, i.e. up to 50 μg of sialic acids injected and the detection limit, estimated as twice the base line noise, was 10 ng for both of SA.

The various glycoconjugates present in blood serum contain sialic acids. Analysis of SA in rat blood serum is given in Fig. 2B. The major sialic acid is Neu5Ac, whereas Neu5Gc content is 5.7 % of total SA, approximately, exhibiting excellent agreement with literature values[24,25] (3-9%). The proposed sample clean-up procedure has been applied to the analysis of six samples of the same rat serum. By injecting equal volumes of the final solution, it was found that the estimated levels of Neu5Ac and Neu5Gc were 1.8% and 2.1% of the target values, respectively, suggesting an accurate and repeatable clean-up procedure. The chromatogram obtained by analysis of SA in C57BI mouse lungs is given in Fig. 3. The Neu5Ac and Neu5Gc contents were 313 ± 44 and 1417 ± 198 μg/g, respectively. It is worth noting that this is the first time that Neu5Gc has ever been described as the major sialic acid in glycoconjugates.

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REFERENCES

1. A. Rosenberg, C. Schengrund, **Biological Roles of Sialic Acids**, Plenum, New York, (1976).

2. R. Schauer, Trends Biochem., 7, 357-360 (1985).

3. I. E. Horgan, Clin. Chim. Acta, **118**, 327-331 (1982).

4. Z. Hrncir, V. Pidrman, M. Tichy, A. Hamet, Vnitr. Lek., **21**, 436-439 (1975).

5. M. Succari, M. J. Foglietti, F. Percheron, Pathol. Biol., **30**, 151-154 (1982).

6. H. K. B. Silver, K. A. Karim, E. L. Archibald, F. A. Salinas, Cancer Res., **39**, 5036 (1979).

7. H. K. B. Silver, K. A. Karim, F. A. Salinas, Br. J. Cancer, **41**, 745-750 (1980).

8. H. A. Harvey, A. Lipton, D. White, Cancer, **47**, 324-327 (1981).

9. A. Colli, G. Buccino, M. Cocciolo, R. Parravicini, F. Mariani, G. Scaltrini, Cancer, **63**, 912-916 (1989).

10. A. Lagana, A. Marino, G. Fago, B. Pardo Martinez, Anal. Chim. Acta, **306**, 65-71 (1995).

11. H. Higashi, Y. Hirabayashi, Y. Fukui, M. Naiki, M. Matsumoto, S. Veda, S. Kato, Cancer Res., 45, 3796-3802 (1985).

12. M. Naiki, H. Higashi, Adv. Exp. Med. Biol., **152**, 445-456 (1982).

13. T. Kawai, A. Kato, H. Higashi, S. Kato, M. Naiki, Cancer Res., **51**, 1242-1246 (1991).

14. A. K. Shukla, R. Shauer, Anal. Biochem., **158**, 158-164 (1986).

15. N. K. Karamanos, B. Wikström, C. A. Antonopoulos, A. Hjerpe, J. Chromatogr., **503**, 421-429 (1990).

16. S. Hara, M. Yamaguchi, Y. Takemori, K. Furuhata, H. Ogura, M. Nakamura, Anal. Biochem., **179**, 162-166 (1989).

17. Z. E. Rassi, Y. Mechref, Electrophoresis, **17**, 275-301 (1996).

18. A. K. Shukla, N. Scholz, E. H. Reimerdes, R. Schauer, Anal. Biochem., **122**, 78-82 (1982).

19. H. K. B. Silver, K. A. Karim, M. J. Gray, F. P. Salinas, J. Chromatogr., **224**, 381-388 (1981).

20. J. R. Scocca, Anal. Biochem., **156**, 61-66 (1986).

21. R. Schauer, Adv. Carbohydr. Chem., **40**, 131 (1982).

22. A. Varki, Anal. Biochem., **137**, 236-247 (1984).

23. G. Reuter, R. Schauer, "Determination of Sialic Acids," in **Guide to Techniques in Glycobiology**, W. J. Lennarz, G. W. Hart, Eds., Academic Press, New York, 1994, pp 176-177.

24. A. Lagana, A. Marino, G. Fago, B. P. Martinez, Anal. Biochem., **215**, 266-272 (1993).

25. S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem., **164**, 138-145 (1987).

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