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Quantitative analysis of sugar constituents of glycoproteins by capillary electrophoresis

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A method for quantitative analysis of monosaccharides including N-acetylneuraminic acid derived from sialic acid-containing oligosaccharides and glycoproteins is presented. The analysis is based on the combination of chemical and enzymatic methods coupled with capillary electrophoretic (CE) separation and laser-induced fluorescence (LIF) detection. The present method utilizes a simplified acid hydrolysis procedure consisting of mild hydrolysis (0.1 M TFA) to release sialic acid and strong acid hydrolysis (2.0 N TFA) to produce amino and neutral sugars. Amino sugars released from strong acid hydrolysis of oligosaccharides and glycoproteins were reacetylated and derivatized with 8-aminopyrene-1,3,6-trisulfonate (APTS) along with neutral sugars in the presence of sodium cyanoborohydride to yield quantitatively the highly stable fluorescent APTS adducts. N-acetylneuraminic acid (Neu5Ac), a major component of most mammalian glycoproteins, was converted in a fast specific reaction by the action of neuraminic acid aldolase (N-acylneuraminate pyruvate-lyase EC 4.1.3.3) to N-acetylmannosamine (ManNAc) and pyruvate. ManNAc was then derivatized with APTS in the same manner as the other monosaccharides. This method was demonstrated for the quantitation of pure Neu5Ac and the species derived from mild acid hydrolysis of 6′-sialyl-N-acetyllactosamine and bovine fetuin glycan. Quantitative recovery of the N-acetylmannosamine was obtained from a known amount of Neu5Ac in a mixture of seven other monosaccharides or from the sialylated oligosaccharides occurring in glycoproteins. The sequence of procedures consists of acid hydrolysis, enzymatic conversion and APTS derivatization which produced quantitative recovery of APTS-monosaccharide adducts. The detection limits for sugars derivatized with APTS and detected by CE-LIF are 100 pmol for Neu5Ac and 50 pmol for the other sugars.

Key words: 8-aminopyrene-1,3,6-trisulfonic acid (APTS)/N-acetylneuraminic acid/monosaccharides/fetuin/capillary electrophoresis with laser-induced fluorescence detection

Introduction

The acid catalysis of the reductive amination of reducing sugars with 8-aminopyrene-1,3,6-trisulfonate (APTS) (Evangelista *et al*., 1996) has been investigated recently in order to improve the labeling efficiency for N-acetylamino sugars, which are usually at the reducing end of the O- and N-linked oligosaccharides in most glycoproteins. N-Acetylamino sugars have previously been found to produce lower yields of detectable adducts in reductive amination with 2-aminopyridine (Takemoto *et al*., 1985, Honda *et al*., 1989). By using citric acid as catalyst, reductive amination of N-acetylamino sugars along with other neutral monosaccharides could be recovered quantitatively (Evangelista *et al*., 1996). Typically, for monosaccharide composition analysis, oligosaccharides or glycoproteins are hydrolyzed in acidic medium to produce the constituent sugars which are then labeled with a chromophore or fluorophore at the reducing termini by reductive amination. The reductive amination scheme for derivatization with APTS is shown in Figure 1. The stoichiometry of labeling is one molecule of fluorophore per monosaccharide. Under milder acid hydrolytic conditions, sialic acid may be released from the glycan and analyzed separately from the other sugars.

The methods currently employed for sialic acid analysis are colorimetry, thin layer chromatography (TLC), gas chromatography, and GC-mass spectrometry (GC/MS) (Reuter and Schauer, 1984). Due to lack of reducing ends, sialic acids are unreactive under reductive amination conditions, the most common derivation reaction for carbohydrates. Analytical methods for sialic acids involving derivatization with 1,2-diamino-4,5-dimethylenedioxybenzene (Hara *et al*., 1989) and ortho-phenylenediamine (Anumula, 1995) were reported recently. The derivatization adducts were analyzed by liquid chromatography (LC) with fluorescence detection. A labeling reaction for capillary electrophoretic analysis involving condensation of the carboxylic acid group of N-acetylneuraminic acid with the amino group of 7-aminonaphthalene-1,3-disulfonic acid using a water-soluble carbodiimide was reported recently (Mechref and El Rassi, 1996).

We reported previously on the quantitation of monosaccharides commonly found in glycoproteins by a simple derivatization procedure using excess 8-aminopyrene-1,3,6-trisulfonate (APTS) and sodium cyanoborohydride in citric acid solution (Evangelista *et al*., 1996). The resulting APTS-monosaccharide adducts were analyzed by capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. Here we report an expanded method which includes the analysis of N-acetylneuraminic acid for the complete composition analysis of monosaccharides occurring in glycoproteins. The method is based on an enzymatically catalyzed conversion of N-acetylneuraminic acid to N-acetylmannosamine and pyruvic acid (Uchida *et al*., 1984). The resulting N-acetyl-

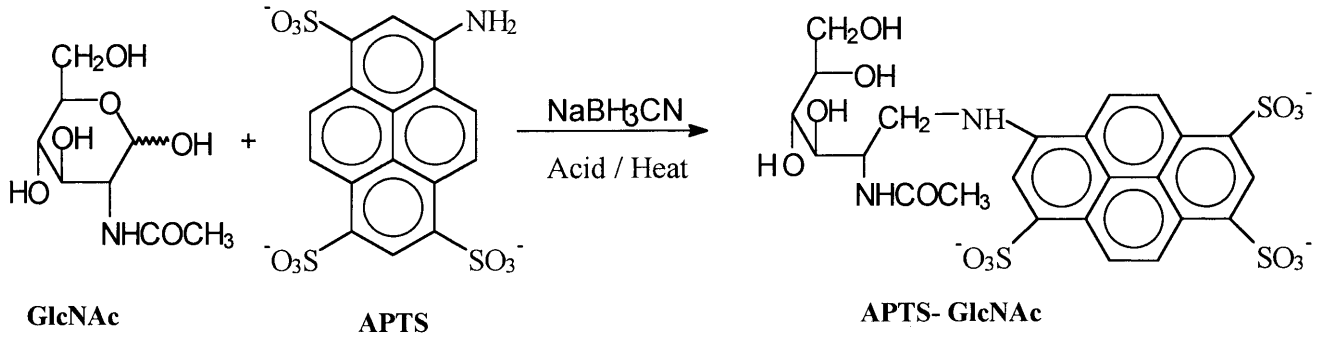

Fig. 1. Reductive amination of N-acetylglucosamine with APTS.

mannosamine was quantitatively derivatized with APTS, and the fluorescent adducts were analyzed by CE-LIF.

Results and discussion

Reductive amination of sugars with APTS from three different sources

Three different APTS reagents were tested for derivatization of a dried standard mixture of 50 pmol of each of six monosaccharides (GalNAc, GlcNAc, Man, Glc, Fuc, and Gal) (Figure 2). The peak which consistently appeared between GalNAc and GlcNAc in all the reagent blanks and the sugar derivatization mixtures was shown to be derived from an impurity present in the $NaBH_3CN$/THF solution since replacement of THF with DMSO or water did not yield the same species. The results show that the Beckman APTS/citric acid/$NaBH_3CN$ blank produced no peak larger than 20% of the 50 pmol sugar peaks except for peak X which was derived from the $NaBH_3CN$/THF solution. The APTS/citric acid/$NaBH_3CN$ blanks from the other commercial APTS reagents produced major peaks which interfered significantly with the analysis of the 50 pmol sugar mixture. Functional testing of APTS for both monosaccharide and oligosaccharide derivatization indicated the importance of reagent purity. Using 200 nmol of APTS to derivatize a sample containing 50 pmol of a monosaccharide, the amount of any fluorescent impurity migrating at the proximity of the adduct should be minimized at less than 10 pmol. Thus, the purity of the APTS reagent should be greater than 99.99% to allow detection of subnanomole amounts of monosaccharides. The High-Purity APTS (Beckman Instruments, Inc., Fullerton, CA), prepared by repeated silica gel chromatographic procedure, allows derivatization and detection of reducing sugars at amounts substantially less than 100 pmol.

Reductive amination of seven monosaccharides with APTS in citric acid

Mixtures of reducing sugars containing 10 pmol to 10 nmol of each monosaccharide were derivatized by reductive amination with excess APTS and sodium cyanoborohydride (200 and 1000 nmol, respectively). The CE-LIF electropherograms of the diluted reaction mixtures showed baseline separation of derivatives of seven monosaccharides using 120 mM borate pH 10.2 in a 20 μm × 27 cm fused silica capillary, shown in Figure 3A. The resulting CE-LIF analyses produced signals which were linearly proportional to the amount of sugars varying from 10 pmol to 10 nmol with a correlation coefficient of 0.995 or higher, as shown in Figure 3B. It can be seen that the peak areas for the sugar with the highest CE-LIF signal, galactose, is about 24% higher than the corresponding values for N-acetylgalactosamine, the sugar which produces the lowest signal. This difference is much smaller than those observed with other reported methods such as HPLC of sugars derivatized with 2-aminopyridine (Takemoto *et al*., 1985; Honda *et al*., 1989) where the signal between N-acetylamino sugars and other aldoses can vary by as much as 3-fold. The use of citric acid as catalyst for reductive amination instead of the commonly used acetic acid substantially reduced the differences in reactivity among the sugars as we previously reported (Evangelista *et al*., 1996).

Analysis of N-acetylneuraminic acid

Sialic acids are a family of 9-carbon carboxylated monosaccharides usually found in glycoproteins as the terminal sugar of oligosaccharides. N-Acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactopyranos-1-onic acid) (Neu5Ac), by far the most common and abundant species, is believed to be the biosynthetic precursor for all the other family of the sialic acids (Varki, 1992). The enzyme N-acetylneuraminic acid aldolase catalyzes the retro-Aldol reaction of N-acetylneuraminic acid resulting in the formation of N-acetylmannosamine (ManNAc) and pyruvic acid. After Speed Vac drying, the enzymatic reaction mixture was derivatized with APTS to yield APTS-ManNAc which was proven to be identical to an authentic sample of the APTS-ManNAc. Figure 4A shows the CE-LIF analysis of products of aldolase-treated eight-monosaccharide mixtures (GalNAc, GlcNAc, Man, Glc, Fuc, Xyl, Gal, and Neu5Ac) containing 0.1–10 nmol of each sugar after APTS derivatization. The results show that Neu5Ac was quantitatively converted to ManNAc whose APTS derivative migrated between the GalNAc and the GlcNAc adducts. The results show that the aldolase reaction had no effect on the other seven sugars present in the mixture, an example of specificity that is typical of enzymatic reactions. The species migrating immediately before the APTS-ManNAc was identified as a contaminant present in $NaBH_3CN$/THF solution that is reactive to APTS under reductive amination conditions. In spite of this contaminant species in the reaction mixture, the recovery and quantitation of N-acetylneuraminic acid by the combination of enzymatic and chemical derivatization could be readily performed quantitatively, as shown in Figure 4A. The CE-LIF signals were linearly proportional to the amounts of sugar derivatized with correlation coefficients greater than 0.996 (Figure 4B).

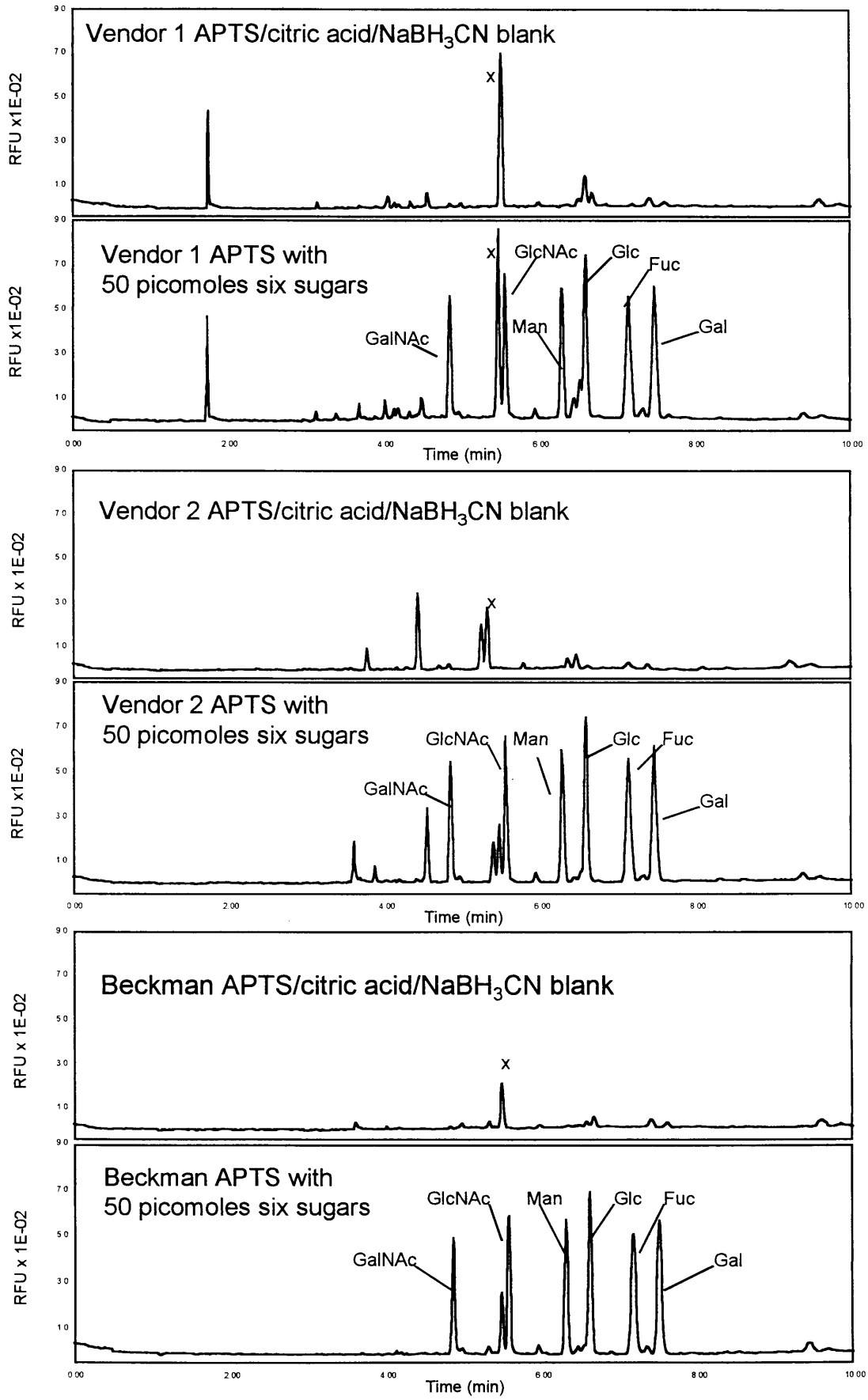

Fig. 2. Diluted reagent blanks (APTS/citric acid/$NaBH_3CN$) and 50 pmol six-sugar derivatization mixture using three commercial APTS reagents. Conditions: 240 mM borate pH 9.0, 20 kV/28 μA, 25 μm × 27 cm, 5 s injection (0.5 psi), 488 ex/520 em, outlet = cathode.

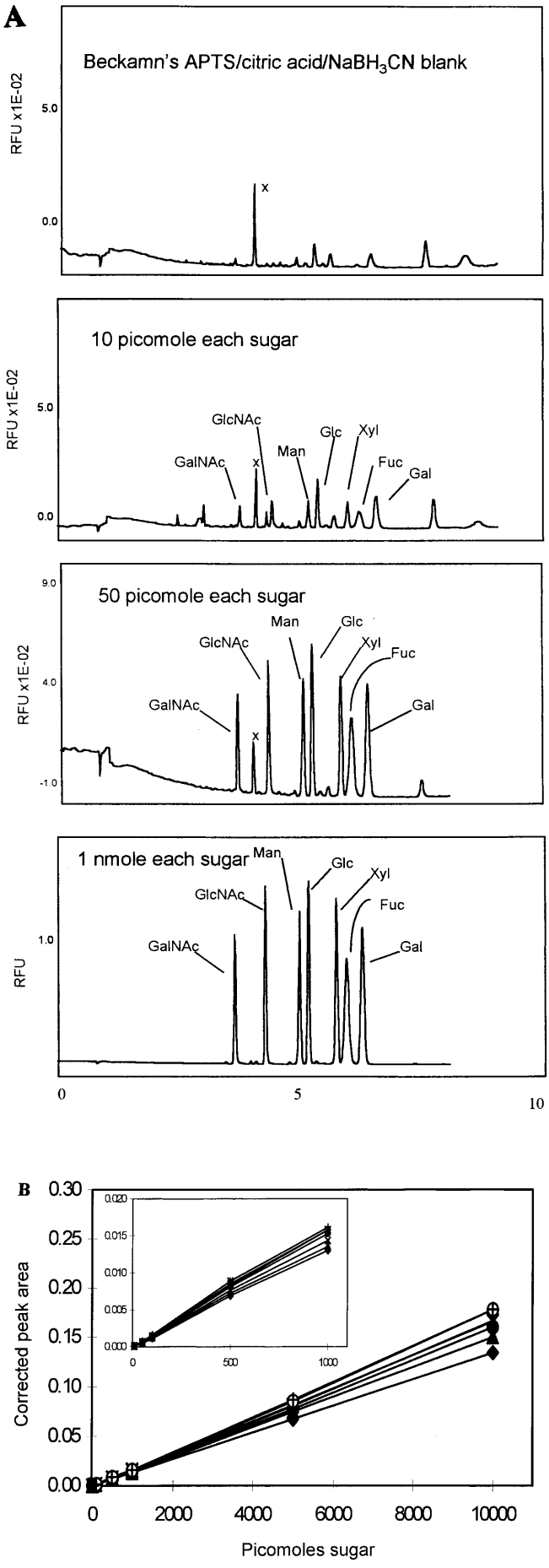

Fig. 3. (**A**) Electropherograms of APTS derivatives of mixtures of seven monosaccharides from 10 pmol to 5 nmol. Conditions: 120 mM borate pH 10.2, 25 kV/22 μA, 20 μm × 25 cm, 488 nm ex/520 nm em. (**B**) Linearity of CE-LIF signal as a function of amount of sugar derivatized. +, Fuc; open circles, Gal; asterisks, Glc; solid circles, Xyl; solid triangles, GlcNAc; multiplication sign, Man; diamonds, GalNAc.

Acid hydrolysis of 6′-sialyl-N-acetyllactosamine, Lewis x trisaccharide, and fetuin

Mild acid hydrolysis (0.1 N TFA/80°C/1 h) of 6′-sialyl-N-acetyllactosamine resulted in the formation of N-acetylneuraminic acid and N-acetyllactosamine. Before the enzyme treatment, N-acetyllactosamine is seen as the only product of the mild hydrolysis (Figure 5A) because the other sugar produced, N-acetylneuraminic acid, is unreactive under the reductive amination conditions. Figure 5B shows that the treatment of the mild acid hydrolysate with *E.coli* N-acetylneuraminic acid aldolase resulted in the formation of equal molar amounts of the N-acetyllactosamine and ManNAc. The by-product of the aldolase reaction, pyruvic acid was not converted to the APTS adduct. Pyruvic acid and N-acetylneuraminic acid, both α-keto acids, are not reactive under the reductive amination conditions.

The results of CE-LIF analysis of the products of strong acid hydrolysis (2.0 N TFA/100°C/5 h) showed that Gal was recovered fully while GlcNAc appeared to be mostly converted to $GlcNH_2$ which presumably reacted poorly APTS as seen in Figure 5C. Reacetylation of the hydrolysate followed by reductive amination with APTS showed full recovery of GlcNAc (Figure 5D). Treatment of the dried product of the strong acid hydrolysis, either before or after reacetylation, with *E.coli* N-acetylneuraminic acid aldolase, yielded an electropherogram (data not shown) showing no ManNAc peak, indicating that N-acetylneuraminic acid was destroyed during the strong acid hydrolysis.

The strong acid hydrolysis of Lewis x yielded Gal, Fuc and glucosamine which produced only a small signal as in the case of 6′-sialyl-N-acetyllactosamine. Reacetylation of the mixture produced quantitative recovery of GlcNAc. Figure 6 shows that the three constituent sugars of Lewis x (GlcNAc, Fuc and Gal) produce peaks of equal intensity. The CE-LIF results show that the recovery of sugars from both the mild and strong acid hydrolysis of 6′-sialyl-N-acetyllactosamine and 3′-sialylactose is essentially quantitative by the present procedure.

The APTS-derivatized aldolase-treated product of the mild acid hydrolysis of bovine fetuin shows only the ManNAc peak (Figure 7A). This result demonstrates specific sialic acid release and resistance of the other endoglycosidic bonds and the protein–carbohydrate linkage towards hydrolytic cleavage during the mild acid hydrolysis. The analysis of APTS adducts of the reacetylated sugars resulting from strong acid hydrolysis of fetuin (Figure 7B) show peaks corresponding to GalNAc, GlcNAc, Man, and Gal. The percentage monosaccharide composition of the fetuin glycan was calculated based on the corrected peak areas relative to the known amounts of the six-sugar standard (Figure 7R) assuming that the protein hydrolysate does not affect the derivatization reaction. This resulted in values which agree well with those reported using other analytical methods (Table I).

It has been reported that mild acid hydrolysis can also result in the loss of fucose linked to GlcNAc (α-1,6, α-1,3, and α-1,4 linkage). To test for release of fucose during mild acid hydrolysis step, sialyl-Lewis^x and sialyl-Lewis^a were subjected to the 0.1 M TFA/1 h/80°C treatment. The analysis of the hydrolysates show

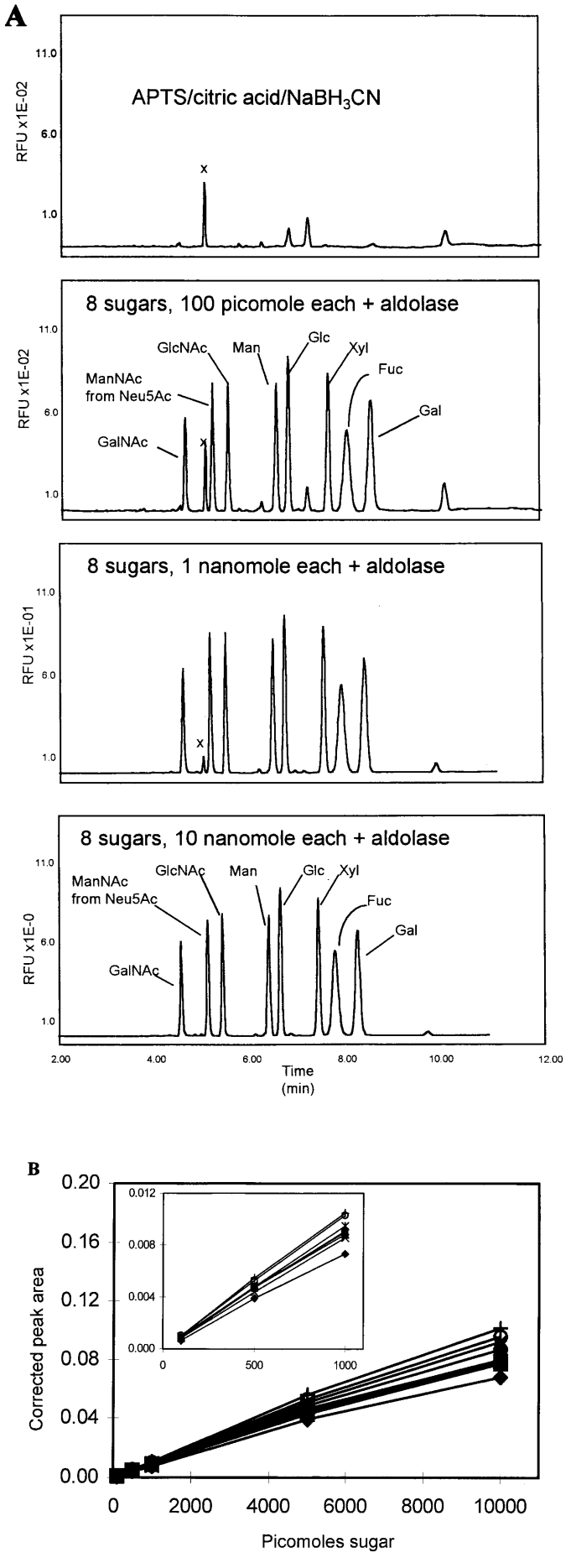

Fig. 4. (**A**) Electropherograms of APTS derivatives of aldolase-treated mixture of N-acetylneuraminic acid and seven aldoses from 100 pmol to 10 pmol. Conditions: 120 mM borate pH 10.2, 25 kV/22 μA, 20 μm × 25 cm, 488 nm ex/520 nm em. (**B**) Linearity of CE-LIF signal as a function of sugar derivatized. +, Fuc; open circles, Gal; asterisks, Glc; solid circles, Xyl; triangles, GlcNAc; multiplication signs, Man; solid squares, ManNAc from Neu5Ac; diamonds, GalNAc.

that the release of sialic acid as well as fucose is essentially complete. Defucosylation of the glycan during the desialylation step does not affect the quantitation of Neu5Ac since the MaNAc-APTS peak used for Neu5Ac quantitation has a much shorter migration time than the Fuc-APTS peak. The quantitation of fucose is performed only during the analysis of the strong acid hydrolysate. Since fetuin does not contain fucose, analysis of the mild acid hydrolysate of this glycoprotein shows only the ManNAc-APTS peak (Figure 7A).

We have developed a system using two internal reference markers to accurately identify the sugars in a sample as compared to standards. The sugar analytes produce APTS adducts with peaks between the two reference markers. Each of the sugar analytes would have its own relative migration time calculated from the reference marker migration times. The peak for one of the internal reference markers, the APTS adduct of glycoaldehyde

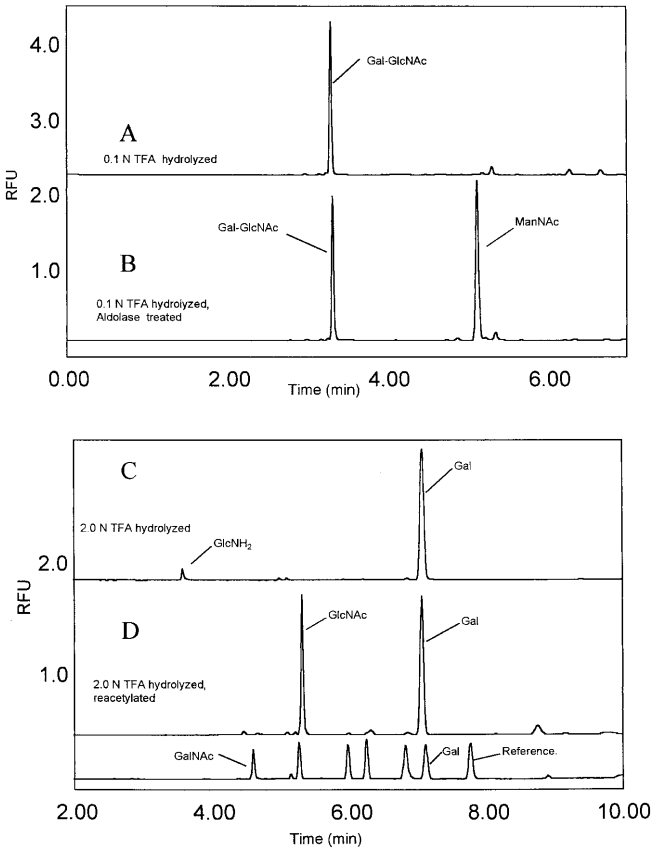

Fig. 5. (**A**) Mild acid hydrolysis product of 6′-sialyllactosamine derivatized with APTS. (**B**) Aldolase-treated mild acid hydrolysis product derivatized with APTS. (**C**) Strong acid hydrolysis product of 6-sialyllactosamine, no reacetylation. (**D**) Same as (**C**), but with reacetylation. Conditions: same as Figure 2.

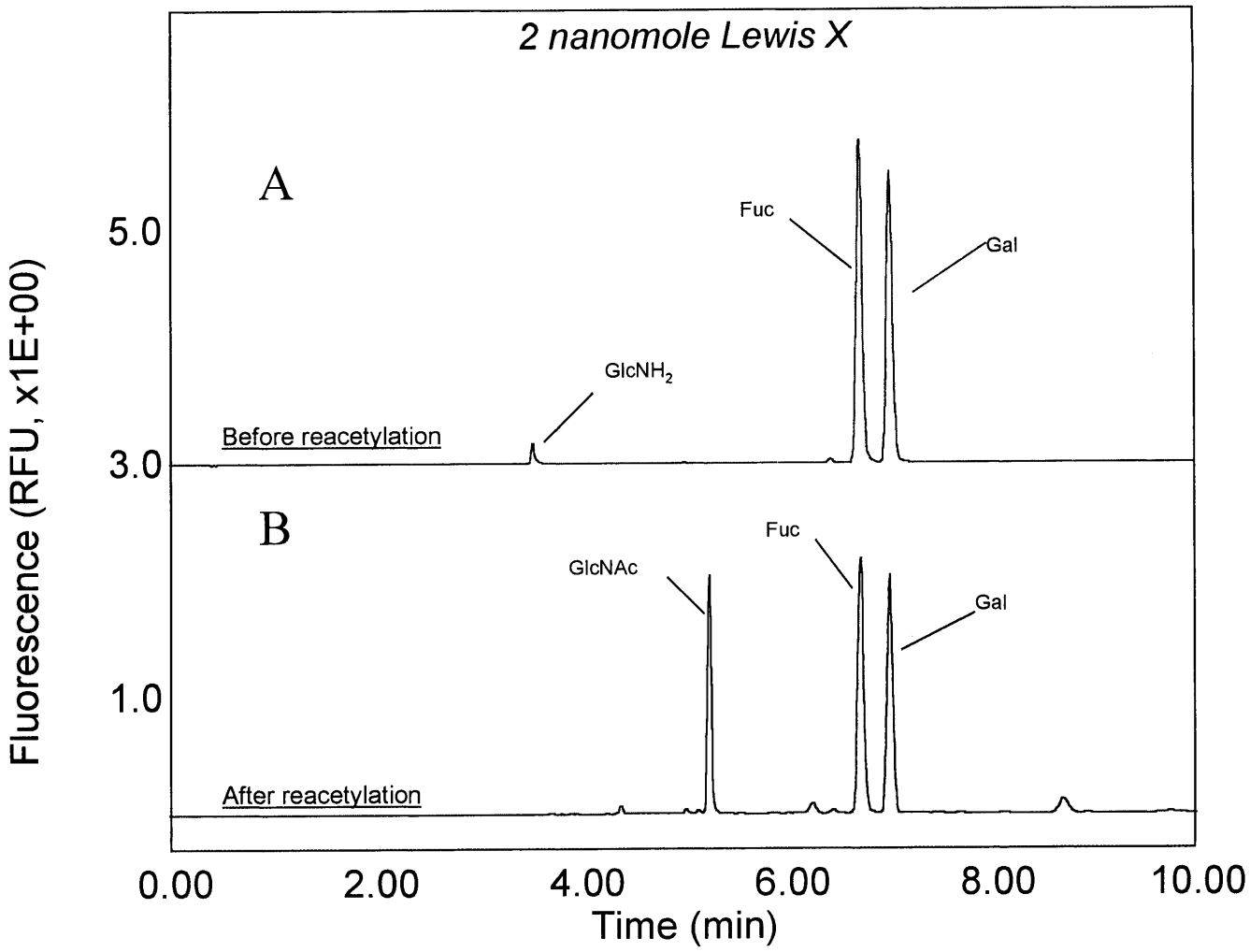

Fig. 6. Strong acid hydrolysis product of Lewis x trisaccharide before (**A**) and after reacetylation (**B**). Conditions: same as Figure 2.

which elutes after the glycoprotein monosaccharide derivatives is shown at the bottom of the electropherograms in Figures 5 and 7.

Table I. Monosaccharide composition of fetuin (moles of sugar per mole of fetuin (MW: 36,354))

Monosaccharide	HPLC[a]	ABA[b]	OPD[c]	APTS, CE-LIF
N-Acetylglucosamine (GlcNAc)	13.54	16.21		14.5
N-Acetylgalatosamine (GalNaAc)	1.85	2.21		1.73
Galactose (Gal)	9.71	11.64		10.9
Mannose (Man)	7.21	8.63		8.0
Neuraminic acid (Neu5Ac)	?		12.5	18.8

[a]Hardy *et al.*, 1988.
[b]Anumula, 1995.
[c]Anumula, 1995.

In summary, the above results show that the conversion efficiencies of each of the steps of the present monosaccharide analysis consisting of acid hydrolysis, enzymatic conversion and chemical derivatization are nearly quantitative for N-acetylneuraminic acid and the other reducing monosaccharides present in glycoproteins.

Materials and methods

Materials

High-purity 8-aminopyrene-1,3,6-trisulfonate (APTS) was obtained from Beckman Instruments, Inc., Fullerton, CA. APTS was also purchased from Lambda Fluorescence Company, Pleasant Gap, PA, and from Molecular Probes, Eugene, OR. Sodium cyanoborohydride, 1.0 M solution in tetrahydrofuran (THF) and trifluoroacetic acid (TFA) were obtained from Aldrich Chemical Co., Milwaukee, WI. Buffer components, citric acid, bovine fetuin, *E.coli* N-acetylneuraminic acid aldolase, N-acetylneuraminic acid and other monosaccharides were purchased from Sigma Chemical Co., St. Louis, MO. 6′-Sialyl-N-acetyllactosamine and Lewis x were purchased from V-Labs, Covington, LA. Maltodextrin M040 was a sample obtained from Grain Processing Corporation, Muscatine, IA.

Methods

Mild acid hydrolysis of oligosaccharides and glycoproteins for release of N-acetylneuraminic acid. To release N-acetylneuraminic

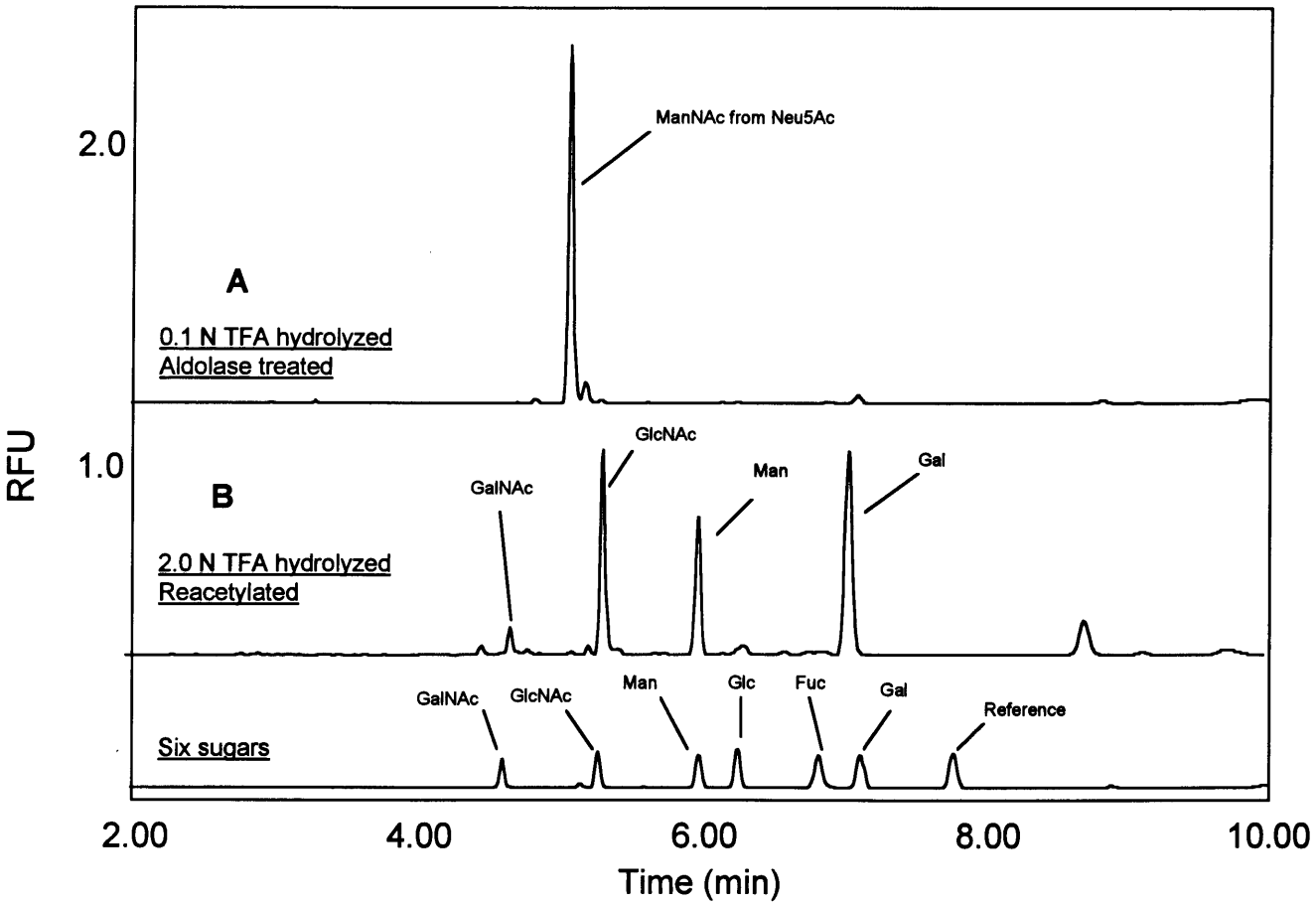

Fig. 7. (**A**) Mild acid hydrolysis product of fetuin after aldose treatment. (**B**) Strong acid hydrolysis of bovine fetuin after reacetylation. Conditions: same as Figure 2.

acid from an oligosaccharide or glycoprotein, a solution of sialylated oligosaccharide (20 nmol) or fetuin (50 μg) was concentrated to dryness on a Speed Vac Concentrator (Savant Instruments) and the residue was dissolved in 100 μl of a 0.1 N TFA in a 500 μl Eppendorf SafeLock conical microcentrifuge tube. The tube was capped tightly and heated in a sand bath at 80°C for 1 h (GlyKo, 1994). Aliquots of the hydrolysates (10 μl) were distributed to 500 μl Eppendorf SafeLock conical microcentrifuge tubes and concentrated to dryness by vacuum centrifugation.

Enzymatic conversion of N-acetylneuraminic acid to N-acetylmannosamine. E.coli N-acetylneuraminic acid aldolase solution (Sigma, 1 μl of 46 U/ml) was added to the sample containing Neu5Ac dissolved in 5 μl 50 mM phosphate buffer pH 7.5. After 45 min incubation at room temperature, the mixture was concentrated to dryness for APTS derivatization.

Strong acid hydrolysis of sialylated oligosaccharides and glycoproteins. For analysis of neutral and N-acetylated sugars, the dried sample containing oligosaccharide (20 nmol) or glycoprotein (fetuin, 50 μg) (produced by Speed Vac concentration) was mixed with 100 μl of a 2.0 N TFA (Hardy *et al*., 1988) in a 500 μl Eppendorf SafeLock conical microcentrifuge tube. The tube was capped tightly and then heated in a sand bath at 100°C for 5 h. Aliquots (10 μl each) containing monosaccharides from 2 nmol of oligosaccharide or 5 μg of glycoprotein were concentrated to dryness for the reacetylation reaction.

Reacetylation of amino sugars. The dried monosaccharide mixture in each tube from the strong acid hydrolysis was redissolved in 5 μl 25 mM carbonate pH 9.5 and then reacetylated by addition of 2 μl acetic anhydride and incubated for 30 min at room temperature. Each mixture was then concentrated to dryness on the Speed Vac concentrator in preparation for reductive amination with APTS.

Derivatization procedures. The derivatization procedure for monosaccharides, including N-acetyl sugars were essentially adapted from the method described previously (Evangelista *et al*., 1996) and modified as follows. Briefly, a dried mixture of sugars from the hydrolysates of glycoconjugates or standard monosaccharide mixture (in a 500 μl Eppendorf SafeLock conical microcentrifuge tube) produced by Speed Vac concentration was derivatized by addition of 2 μl 100 mM APTS in 0.9 M citric acid followed by addition of 1 μl 1.0 M $NaBH_3CN$ in THF. In this standard derivatization procedure, the reaction mixture contains varying amount of sugars and constant concentration of reagents (67 mM APTS, 0.6 M citric acid, and 0.33 M $NaBH_3CN$). The mixture was vortexed, centrifuged and then heated in a water bath at 55°C for 2 h. The resulting reaction mixture was diluted with water to 200 μl and was diluted further 10-fold prior to CE-LIF analysis. A blank sample containing APTS, citric acid, and $NaBH_3CN$ in the 500 μl Eppendorf SafeLock conical microcentrifuge tube was also prepared. We have found that the background reagent blank signal can vary greatly depending on the container used due to impurities extracted from the plastic and

this has a substantial effect on the derivatization detection limit. The 500 µl Eppendorf SafeLock conical microcentrifuge tube produced the lowest background signal with the least interference on the APTS-sugar peaks.

Capillary electrophoretic separations. CE analyses were performed on a P/ACE 2100 equipped with laser-induced fluorescence detector (Beckman Instruments, Inc., Fullerton, CA), and the separation conditions were essentially same as reported previously (Chen and Evangelista, 1995; Evangelista *et al*., 1995). Separations were performed in 20 µm × 27 cm fused silica capillary using 120 mM borate buffer pH 10.2 or in 25 µm × 27 cm fused silica capillary using 240 mM borate buffer pH 9.0. Samples were introduced to the capillary by pressure injection (0.5 psi) for 20 s. Electrophoresis was performed at the voltage shown in each electropherogram. Running buffers were sodium salt of borate at pH 9.0 or 10.2 and the buffer concentration is indicated in each electropherogram.

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