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Comprehensive Profiling of APTS-Labeled N-Glycans Using Capillary Zone Electrophoresis / Laser Induced Fluorescence

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Abstract: In this article, a method for the analysis of N-linked glycans derived from glycoproteins is described. Complex mixtures of APTS-labeled neutral and acidic N-glycans were resolved using capillary zone electrophoresis coupled with laser-induced fluorescence detector. All separations were carried out on a polyacrylamide coated capillary with reduced electroosmotic flow. N-glycan pools released from several standard glycoproteins were analyzed in order to generate a migration time database used for the identification of N-glycans released from more complicated samples. In addition, enzymatic digestions using specific and non-specific exoglycosidases were used to positively identify glycan isomeric structures.

Keywords: Capillary zone electrophoresis; Laser induced fluorescence; Glycoproteins; N-glycans; Glycan profiling.

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Introduction

Glycosylations are the most abundant post-translational modifications observed in human cells, where more than 50 % of all proteins are believed to be glycosylated [1]. The glycosylation of proteins is a well-controlled process that takes place in the endoplasmic

reticulum of a cell, where the elongation and truncation of a core structure generates a wide range of glycans. These varying glycan stuctures (known as protein glycoforms) define glycoprotein functions and activities [2,3].

Glycosylated proteins play important roles in cell-to-cell interactions, immunosurveillance and a variety of receptor-mediated and specific protein functions through a highly complex repertoire of glycan structures. Accordingly, metabolic disfunctions and disease states may be reflected in the appearance of abnormal glycans or altered quantitative proportions within the glycome. A number of glycoforms associated with certain proteins have been recently identified as putative disease biomarkers and differential glycomic measurements between healthy and disease states may even have a significant clinical diagnostic potential [4,5].

Mass spectrometry (MS) is currently recognized as the ultimate technique for characterization of biomolecules, including glycans released from human glycoproteins [6-9]. Despite the wealth of information provided by the most powerful MS and tandem MS techniques, isomeric structures are not readily identified [10,11]. Therefore, analytical separation techniques provide alternatives for the analysis of glycan pools containing isomeric structures. In this respect, capillary electrophoresis (CE) techniques, especially capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE), are the most promising techniques for separation of glycan isomers [12-15]. As an alternative, hydrophilic interaction liquid chromatography and anion-exchange chromatography are routinely used in glycan analysis [16-18]. However, chromatographic separations are often long (> 60 min) and lack the resolution necessary to identify linkage isomers.

In this paper, capillary zone electrophoresis coupled with LIF detector as a powerful tool for identification of N-linked glycan structures derived from glycoproteins is demonstrated. Complex mixtures of derivatized N-glycans were resolved on a polyacrylamide coated capillary with reduced electroosmotic flow. To detect N-glycans using high sensitive LIF detection, a highly fluorescent dye 1-aminopyrene-3,6,8-trisulfonate (APTS) as a derivatization reagent was attached to the reducing end of oligosaccharide molecule through reductive amination [15]. Glycan structure identification was elucidated using a combination of strategies, including comparison with standard glycan libraries and the enzymatic digestions of N-linked glycan pools using specific and non-specific exoglycosidases.

Experimental

Chemicals and Reagents

High purity 1-aminopyrene-3,6,8-trisulfonate (APTS) was purchased from *Beckman Coulter* (Fullerton, CA, USA). Citric acid, ammonium bicarbonate, trifluroacetic acid (TFA), trifluoroethanol (TFE), sodium cyanoborohydride, dimethylsulfoxide (DMSO), Tris-HCl, γ-methacryloxypropyltrimethoxysilane, methylene chloride, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine, sialidase from *Arthrobacter ureafaciens* (EC 3.2.1.18), ribonuclease B, fetuin, asialofetuin, human and bovine α_1-acid glycoproteins, and ovalbumin were all acquired from *Sigma-Aldrich* (St. Louis, MO, USA). Acrylamide, ammonium persulfate, sodium cyanoborohydride, dithiothreitol (DTT) and iodoacetamide (IAM) were purchased from *Bio-Rad Laboratories* (Hercules, CA, USA). Acetonitrile (ACN) and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). N-glycosidase F (PNGase F) from *Chryseobacterium meningosepticum* (EC 3.2.2.18) was received from *Northstar Bioproducts* (East Falmouth, MA, USA). The enzymes α2-3 specific sialidase (EC 3.2.1.18) from *Streptococcus pneumoniae*, β-galactosidase (EC 3.2.1.23) from *Streptococcus pneumoniae* and β1-3 specific galactosidase (EC 3.2.1.23) from *Xanthomonas manihotis*, in addition to the glycan standards 6'-sialyllactose, hybrid-type glycan with bisecting GlcNAc, NGA2, NGA2F, NGA2FB, NGA3, NA2FB and NA4 were all obtained from *Prozyme* (Hayward, CA, USA).

Throughout the experimental work, all solutions were prepared from doubly deionized water obtained by passing through a laboratory cartridge purifier Milli-Q system (Millipore; Billerica, MA, USA).

Instrumentation

CE System. All experiments were carried out with the P/ACE MDQ Capillary Electrophoresis System from *Beckman Coulter* (Fullerton, CA, USA) coupled with LIF detector using a 488 nm argon-ion laser. The instrument detection system was set for a dynamic range of 100 RFU, while the detector digital acquisition was set to high resolution with a sampling rate of 8 Hz. Fused silica capillaries of 60 cm total length, 50 cm effective length and 25 μm I.D., coated with a linear polyacrylamide (PAA), and Tris-HCl buffer (40 mM, pH 6.5) as a working electrolyte were used for all separations.

The separation temperature was set at 22,5 °C, sustained by the internal coolant system. Samples were stored in a refrigerated sample compartment at 4 °C. Voltage -22.5 kV was applied, while the samples were injected hydrodynamically at 0.4 psi pressure for 0.1 min.

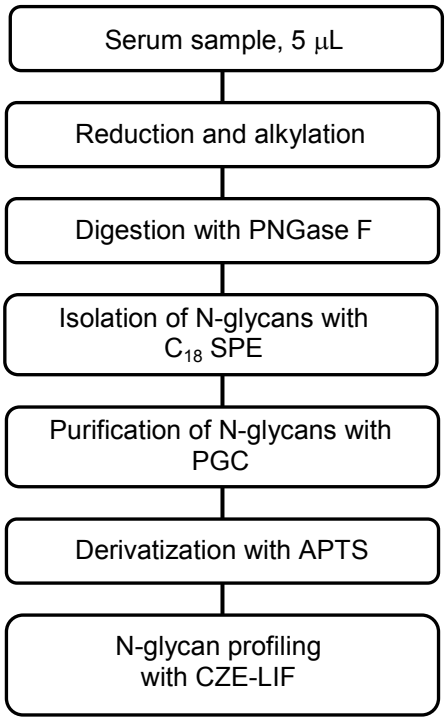

Fig. 1: *Procedure for the isolation and derivatization of N-glycans cleaved from glycoproteins occurring in human blood serum.*

Capillary Preparation. The fused silica capillaries, purchased from *Polymicro Technologies* (Phoenix, AZ, USA) were coated with a linear polyacrylamide according to a modified version of Hjerten's procedure [19,20]. The capillary inner walls were washed with 1.0 M sodium hydroxide aqueous solution for 1 hour, followed with consecutive washes with water and methanol for 15 min. each. The capillary inner walls were then reacted with Bind-Silane, γ-methacryloxypropyltrimethoxysilane solution (prepared by dissolving a 45-μL aliquot in 1.5 mL of methylene chloride containing a 1.7-μL aliquot of acetic acid) for 45 min. Following this step, the capillaries were flushed with methanol and water for 15 min. each. The capillary inner walls were then reacted with 2.4% (w/w) acrylamide aqueous solution, containing 1.0 μL/mL N,N,N',N'-tetramethylethylenediamine and a 1-μL aliquot of ammonium persulfate aqueous solution (1.0 mg/mL), for 120 min.

Finally, the surfaces were rinsed with water for 20 min. and dried under a stream of nitrogen for another 20 min. All solutions were infused through the capillary at an inlet pressure of approximately 100 psi.

Glycan Cleavege and Derivatization

Glycans were released from standard glycoproteins and blood serum samples using an enzymatic cleavage protocol, as illustrated in Fig. 1 and described previously [21]. Briefly, dried sample was resuspended in 100 μL of 50 mM phosphate buffer (pH = 7.0) containing 0.1% β-mercaptoethanol. The solution was then denatured by heating at 95º C for 5 min., and allowed to cool to room temperature prior to the addition of a 0.2-μL aliquot of PNGase F (5 Units/μL). The sample was then incubated at 37º C overnight. Finally, volume of the solution was adjusted to 1 mL by deionized water.

Blood serum samples were reduced and alkylated before the PNGase F cleavage. Briefly, a 5-μL aliquot of serum was evaporated and then resuspended in 25 μL of 25 mM ammonium bicarbonate, 25 μL of TFE and 2.5 μL of 200mM DTT prior to incubation at 60 °C for 45 min. A 10-μL aliquot of 200 mM IAM was then added and allowed to react at room temperature for 1 hour in the dark. Subsequently, a 2.5-μL aliquot of DTT was added to react with the excess of IAM. Next, the reaction mixture was diluted with 300 μL of water and 100 μL of ammonium bicarbonate stock solution to adjust the pH to 7.5 – 8.0, suitable for the enzymatic release of N-glycans using PNGase F.

N-glycans solutions were then applied to both C_{18} Sep-Pak® Cartridges from *Waters* (Milford, MA, USA) and activated charcoal cartridges from *Harvard Apparatus* (Holliston, MA, USA). First, C_{18} cartridge was pre-conditioned with ethanol and deionized water according to the manufacturer procedure. The reaction mixture was circulated through the cartridge 5 times prior to washing with water. Peptides and O-linked glycopeptides were retained on the C_{18} cartridge, while the released N-glycans were collected as eluents. Next, the C_{18} cartridge was washed with 1 mL of deionized water. The combined eluents containing N-glycans were then passed over activated charcoal microcolumns. The columns were pre-conditioned with 1 mL of ACN and 1 mL of 0.1% TFA aqueous solution, according to the manufacturer procedure. After applying a sample, the microcolumn was washed with 1 mL of 0.1% TFA aqueous solution. The samples were then eluted with a 1-mL aliquot of 50% ACN aqueous solution with 0.1% TFA. Finally, the purified glycans were evaporated to dryness using vacuum CentriVap Concentrator from *Labconco Corporation* (Kansas City, MO, USA).

The released N-glycans were derivatized through reductive amination using APTS as a derivatizing reagent, as reported previously [15,20]. Briefly, the released N-glycans were resuspended in a 2-μL aliquot of 0.1 M APTS solution prepared in 0.9 M aqueous citric acid, and a 1-μL aliquot of 1 M sodium cyanoborohydride solution prepared in DMSO. The reaction mixture was then incubated at 57 °C for 2 hrs. The derivatization mixture was then dialyzed extensively overnight in a 1 kDa cut-off MicroDispo dialysis cartridges from *Harvard Apparatus* (Holliston, MA, USA) to reduce/eliminate an excess of the derivatization fluorphore, which could adversely affect a CZE separation. Finally, the dialyzed mixture was lyophilized and resuspended in 25 μL of an internal standard solution (4.0 μg/mL of APTS-derivatized 6′-sialyllactose), which was used to normalize peak migration times and areas. The digestion of derivatized samples using exoglycosidases was performed under the buffer conditions listed in Table I.

Table I: *List of enzymes used for the release and sequencing of N-glycans from glycoproteins.*

Enzyme	Specificity	Optimum pH
PNGase F (*C. meningosepticum*)	N-Linked oligosaccharides	7.5
Sialidase (*S. pneumoniae*)	Release of α2-3 Sialic acid	5.0 – 6.0
Sialidase (*A. ureafaciens*)	Release of α2-6(->3,8) Sialic acid	5.0 – 6.0
α-Fucosidase (*B.kidney*)	Release of α1-6(->2,3,4) Fucose	5.5
β-Galactosidase (*X. manihotis*)	Release of β1-3,6 Galactose	5.0
β-Galactosidase (*S. pneumoniae*)	Release of β1-4 Galactose	5.0
β-N-Acetylhexosaminidase (*S. pneumoniae*)	Release of β1-2,3,4,6 N-Acetylglucosamine	5.0 – 6.0

Results and Discussion

Optimization of Separation Conditions

In previous work [20], capillary zone electrophoresis has been reported as a suitable technique for the separation of certain N-linked glycan structures. To achieve a high resolution and efficiency of CZE separation of N-glycan structures derived from complex glycoprotein samples, our first experiments were focused on the optimization of separation conditions using a mixture of seven N-glycan standards (see Table II).

The influence of capillary dimensions and buffer concentration on separation efficiency and resolution were evaluated in order to maximize the resolution of N-glycans. Separation capillaries with 25 μm inner diameter offered the highest efficiency and resolution, with an efficiency of 715,000 theoretical plates. Moreover, the use of smaller dimensions generates a lower current and Joule heating.

Table II: *Reproducibility data for the analysis of neutral standard N-glycans* (n = 5 for each migration time, t_m)

N-Glycan	**Run-to-Run** t_m (min.)	**Day-to-Day** t_m (min.)	**Capillary-to-Capillary** t_m (min.)	**Batch-to-Batch** t_m (min.)
NGA2	15.51 ± 0.01	15.55 ± 0.02	15.53 ± 0.02	15.51 ± 0.03
NGA2F	16.55 ± 0.01	16.58 ± 0.03	16.56 ± 0.03	16.54 ± 0.03
NGA3	16.70 ± 0.03	16.75 ± 0.05	16.72 ± 0.04	16.72 ± 0.05
NGA2FB	17.05 ± 0.02	17.07 ± 0.05	17.07 ± 0.04	17.05 ± 0.06
Hybrid type with bisecting GlcNAc	17.27 ± 0.03	17.31 ± 0.06	17.30 ± 0.05	17.29 ± 0.08
NA2FB	19.47 ± 0.05	19.51 ± 0.08	19.49 ± 0.07	19.49 ± 0.09
NA4	23.04 ± 0.07	23.1 ± 0.1	23.06 ± 0.09	23.1 ± 0.1

The best separation selectivity of APTS-derivatized standard N-glycans was achieved in 40 mM Tris-HCl buffer (pH = 6.5) using polyacrylamide coated capillary of 60 cm total length (50 cm effective length) while optimum voltage -22.5 kV was applied. Under these conditions, the negatively charged APTS-derivatized glycans migrate in the opposite direction of the electroosmotic flow, which is relatively small (less than 1.0 x 10^{-6} cm^2 V^{-1} s^{-1}). As summarized in Table II, the run-to-run migration time reproducibility is quite high for analyzed linear N-glycans. Over a number of days, there were a few fluctuations in the migration times, while the electrolyte was replenished daily from a stock solution. The capillary-to-capillary variation for a set of five capillaries coated simultaneously with the same coating solution is less than 1% RSD. The batch-to-batch reproducibility is also less than 1% for the capillaries prepared on separate days using freshly prepared coating solutions. By capillary coating with polyacrylamide, the reproducibility of N-glycans separation has been enhanced relative to uncoated fused silica capillaries.

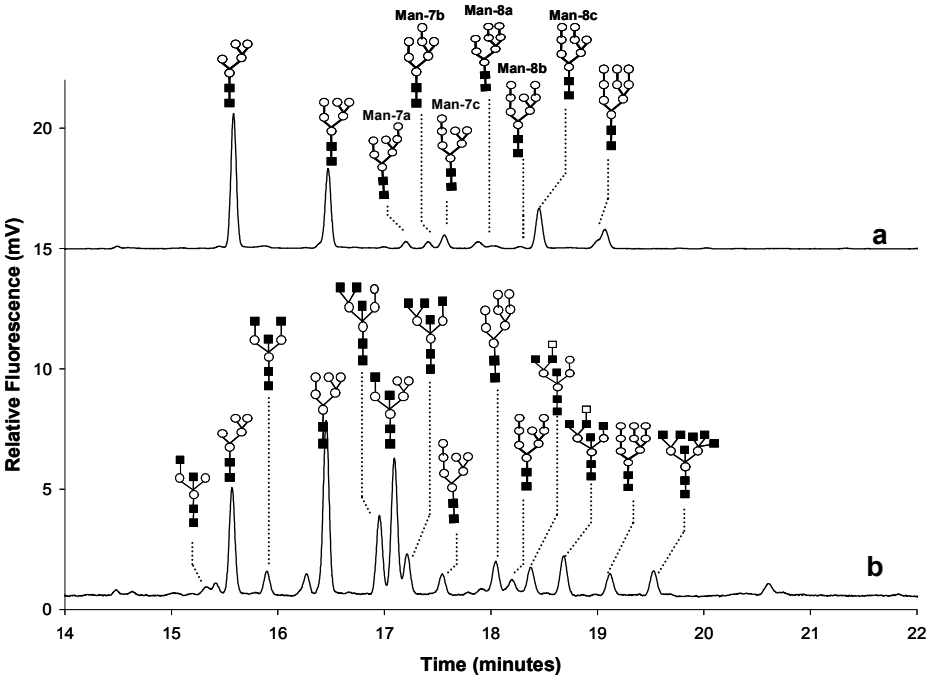

Fig. 2: *Electrophoretic separation of APTS-derivatized N-glycans released from standard glycoproteins.* Legend: **a)** ribonuclease B, **b)** ovalbumin. PAA coated capillary (60 cm × 25 μm I.D.). Tris-HCl buffer, 0.040 mol L^{-1}, pH = 6,50. Voltage -22,5 kV, capillary temperature 22,5 °C, sample injection at 0.4 psi for 0.1 min. LIF detection, argon-ion laser, excitation 488 nm, emission 520 nm. For other details, see *Experimental*.

Analysis of Glycans Released from Standard Glycoproteins

Purified N-glycan pools, released from standard glycoproteins and derivatized with APTS, were analyzed by CZE-LIF in order to generate a migration time database used for an identification of glycan structures released from more complicated real samples. In addition, N-glycan pools released from glycoprotein standards were enzymatically digested using specific and non-specific exoglycosidases to positively identify glycan isomeric structures.

Fig. 2 demonstrates the separation of glycans cleaved from ribonuclease B and ovalbumin. The high-mannose glycans obtained from ribonuclease B contain structures with five to nine mannose units, termed as Man5 – Man9 (glycan structures are listed in the Appendix I). As seen in Fig. 2a, successful resolution of the Man-7 and Man-8 structural isomers was achieved. Fig. 2b shows the separation of ovalbumin glycans, where Man-5, Man-6 and Man-7 structures have similar migration times as seen for high-mannose glycans cleaved from ribonuclease B.

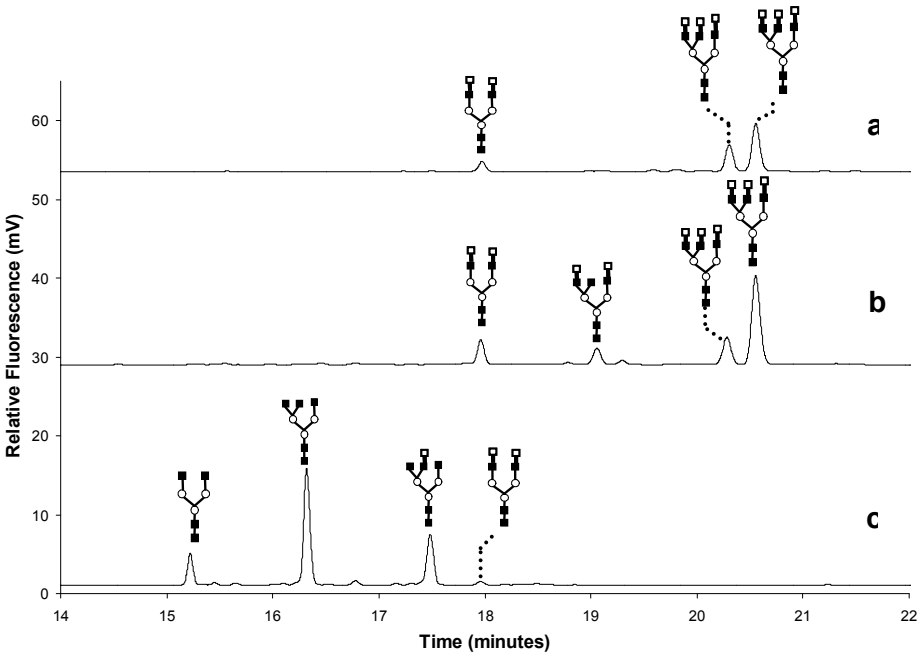

Fig. 3: *Electrophoretic separation of APTS-derivatized N-glycans released from standard glycoproteins.* Legend: **a)** native asialofetuin, **b)** β1-3,6 galactosidase-treated asialofetuin, **c)** β1-4 galactosidase-treated asialofetuin. For separation conditions, see *Fig. 2*.

Fig. 3 demonstrates the separation of asialofetuin glycans with either biantennary or triantennary structure. Two triantennary structures, in which the terminal galactose residue is either β1-4 or β1-3 linked to the adjacent GlcNAc, are known. Positive identification of these two isomers was achieved using galactosidase digestion of asialofetuin (see Fig. 3b & 3c).

Sialylated glycan structures are much more heterogeneous than those of neutral glycans due to the linkage variability of the sialic acid on each terminus. Sialic acids are commonly linked as either α2-3 or α2-6 to the glycan core. Therefore, a biantennary glycan can have four isomers, and a triantennary glycan could have as many as nine isomers. This obviously poses a challenge for the analysis of more complex glycan pools where each structural isomer is possible. The glycan profile of bovine fetuin is shown in Fig. 4a. Next, fetuin was digested with an 2-3-linkage specific sialidase, resulting in a decrease the number of A4 and A3 glycans, while the A2 and A1 glycans increased substantially (see Fig 4b). The separation of fetuin glycans, achieved using digestion with the non-specific sialidase that cleaves α2-3,6 linkages, is shown in Fig. 4c. These glycans are the asialylated structures as seen previously in asialofetuin. The separation of A1 and A2 glycans released from human fibrinogen is illustrated in Fig. 4d . These purified glycans were used to positively identify the α 2-6 linked mono- and disialylated biantennary glycans in fetuin.

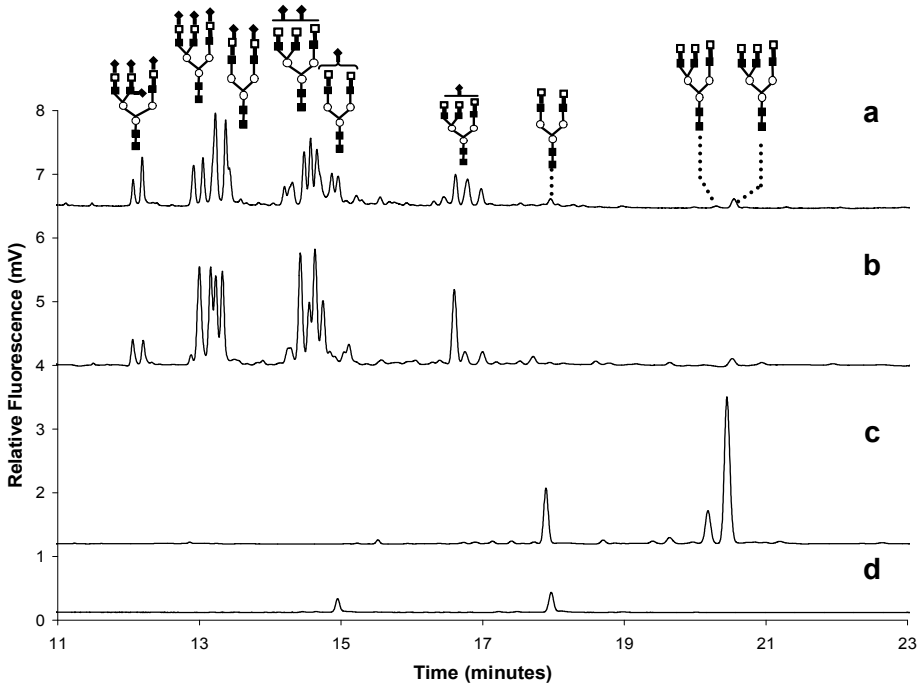

Fig. 4: *Electrophoretic separation of APTS-derivatized N-glycans released from standard glycoproteins.* Legend: **a)** native fetuin, **b)** α2-3 sialidase-treated fetuin, **c)** α2-6(->3,8) sialidase-treated fetuin, **d)** human fibrinogen. For separation conditions, see *Fig. 2*.

Fig. 5a depicts the separation of complex glycans released from α_1-acid glycoprotein, Both sialylated and fucosylated structures are present in analyzed glycan pools. By the same procedure described for fetuin, isomeric glycan structures of α_1-acid glycoprotein were identified using enzymatic sequencing with linkage-specific sialidases and fucosidases. Effect of specific α2-3 linkage sialidase is shown in Fig. 5b. Non specific α2-3,6 sialidase digestion reduces the glycan pool to the corresponding asialylated structures, similar to those found in fetuin, and fucosylated core structures, as seen in Fig. 5c. This figure also illustrates the high resolution capabilities of CZE-LIF technique for a glycan profiling using qualitative comparison.

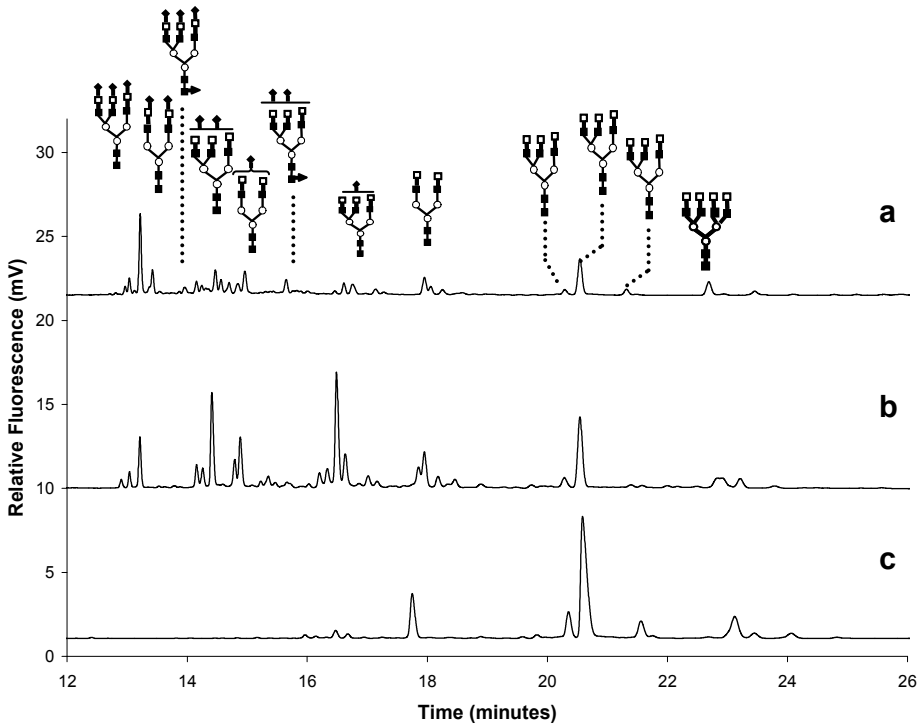

Fig. 5: *Electrophoretic separation of APTS-derivatized N-glycans released from standard glycoproteins.* Legend: **a)** native α_1-acid glycoprotein, **b)** α2-3 sialidase-treated α_1-acid glycoprotein, **c)** α2-6(->3,8) sialidase-treated α_1-acid glycoprotein. For separation conditions, see *Fig. 2*.

Analysis of Glycans Released from Serum Sample

The separation of N-glycans cleaved from purified serum glycoproteins is shown in Fig. 6a. Several classes of glycans can be identified using spatial segregation of the sialylated structures from the neutral glycans. The developed glycan library was applied to identify glycan peaks observed in blood serum electropherograms. Moreover, enzymatic sequencing was carried out to help distinguish specific linkages for positive peak identification. It is apparent from following digestion with specific α2-3 sialidase (see Fig. 6b) that in several glycans the N-acetylneuraminic acid is coupled to the core structure through α2-3-linkage. In addition, all sialic acid residues were cleaved with the non specific α2-3,6 sialidase to determine, which structures in analyzed glycan profile are acidic. As seen in Fig. 6c, the acidic glycans are reduced to their neutral core structures having higher migration times. Because a human blood serum is such a complex sample, not all glycan structures were detemined using strategies described above. However, a nearly complete glycan profile in blood serum sample is demonstrated.

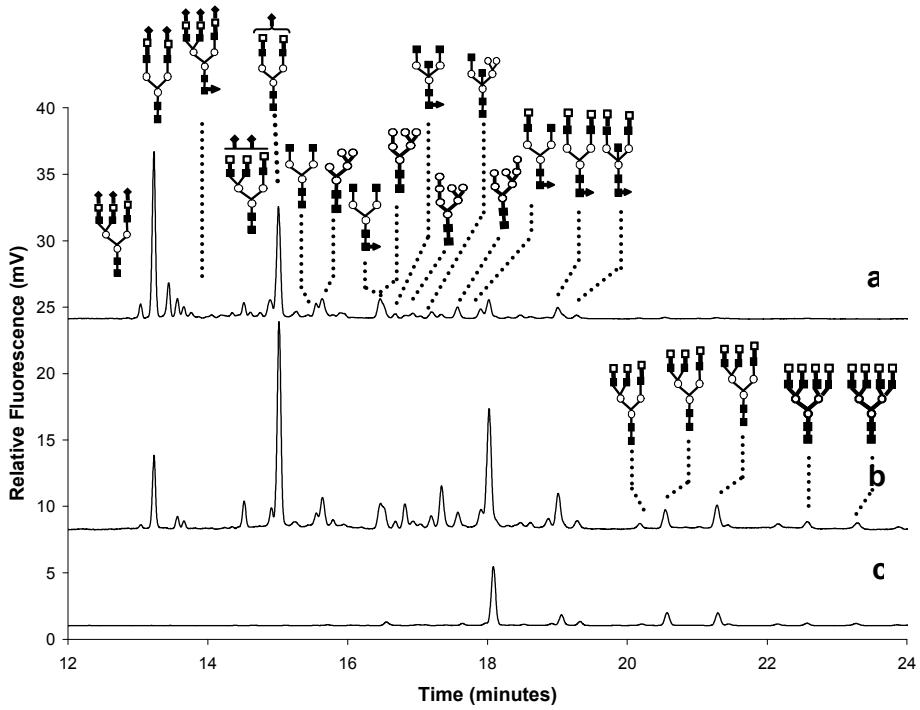

Fig. 6: *Electrophoretic separation of APTS-derivatized N-glycans released from human blood serum.* Legend: **a)** glycans from the native serum, **b)** α2-3 sialidase-treated serum glycans, **c)** α2-6(->3,8) sialidase-treated serum glycans. For separation conditions, see *Fig. 2*.

Conclusions

In this article, a method for the rapid, highly efficient and reproducible profiling of N-linked glycans derived from purified glycoproteins or complex glycan pools has been evaluated. The APTS-derivatized neutral and acidic N-glycans were analyzed using capillary zone electrophoresis coupled with laser-induced fluorescence detector. All separations were carried out on a polyacrylamide coated capillary with reduced electroosmotic flow. The best separation selectivity of APTS-derivatized standard N-glycans was achieved in 40 mM Tris-HCl buffer (pH = 6.5).

Comprehensive glycan structure identification is based on comparison with standard glycan libraries and selective enzymatic digestions of N-linked glycan pools using specific and non-specific exoglycosidases. CZE significantly complements other means for glycan mapping, such as mass spectrometry, due to its ability to resolve structural isomers of N-glycans. Therefore, CZE-LIF can be used as a routine clinical diagnostic tool for glycomic profiling in clinical samples, such as human blood serum.

Acknowledgements

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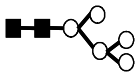
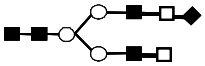
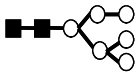
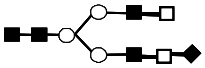
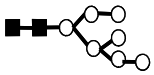
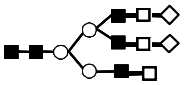
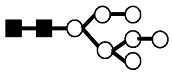
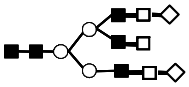
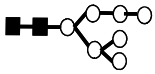
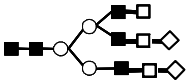
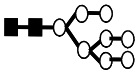
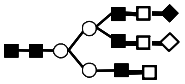
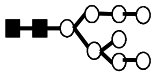
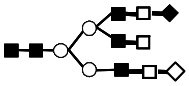
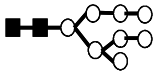
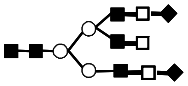
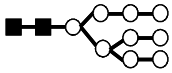
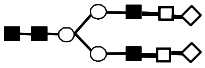
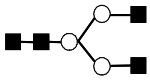
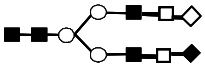
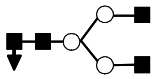
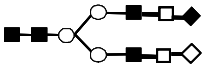
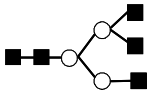
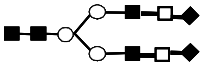
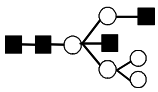
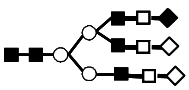
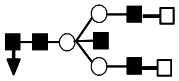
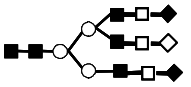
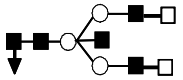
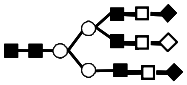
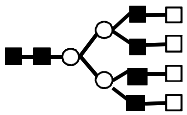
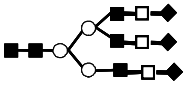
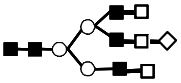
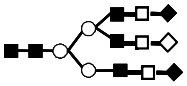
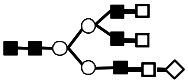
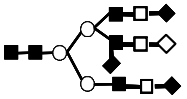
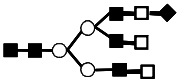
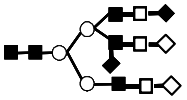
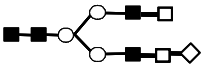
(SEE ALSO OVERLEAF)

Appendix I: *List of N-glycans and their structures.*

Glycan name	Glycan structure	Glycan name	Glycan stucture
Man-5		N-1Db	
Man-6		N-1Dc	
Man-7a		N-2Ta	
Man-7b		N-2Tb	
Man-7c		N-2Tc	
Man -8a		N-2Td	
Man-8b		N-2Te	
Man-8c		N-2Tf	
Man-9		N-2Da	
NGA2		N-2Db	
NGA2F		N-2Dc	
NGA3		N-2Dd	
Hybrid Bisecting GlcNac		N-3Tb	
NA2FB		N-3Tc	

Appendix I: (continued)

Glycan name	Glycan structure	Glycan name	Glycan stucture
NA2FB		N-3Tc	
NA4		N-3Td	
N-1Ta		N-3Te	
N-1Tb		N-4Ta	
N-1Tc		N-4Tb	
N-1Da			

Appendix II: *List of monosaccharide symbols.*

○ D-Mannose (Man)

■ L-Fucose (Fuc)

□ D-Galactose (Gal)

▲ N-Acetyl-D-Glucosamine (GlcNAc)

◇ α2-3 Sialic Acid (NeuNAc)

◆ α2-6 Sialic Acid (NeuNAc)

Author Index

(see also overleaf)

(continued)