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Ultramicroanalysis of Reducing Carbohydrates by Capillary Electrophoresis with Laser-Induced Fluorescence Detection as 7-Nitro-2,1,3-benzoxadiazole-Tagged *N*-Methylglycamine Derivatives[1]

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A method for ultramicroanalysis of carbohydrates using capillary electrophoresis with laser-induced fluorescence detection was developed, based on precapillary conversion to 7-nitro-2,1,3-benzoxadiazole (NBD)-tagged *N*-methylglycamines. Although the derivatization involves two-step reactions, i.e., reductive N-methylamination followed by condensation with NBD-F, they can be carried out in a one-pot fashion and proceed quantitatively within ca. 50 min in total. Since the reaction conditions are mild, it does not cause desialylation. The derivatives can be well separated by capillary electrophoresis and sensitively detected by argon laser-induced fluorescence. It allowed detection of monosaccharides of down to nanomolar concentrations for analytical sample solution, which correspond to the attomole injected amounts, and good linearity was observed over a wide range. It was also successfully applied to analysis of *N*-glycans in a microgram quantity of a glycoprotein. Studies on the cleanup of derivatized product are also described in relation to *N*-glycan analysis. © 2000 Academic Press

Key Words: **CE-LIF; carbohydrates; NBD-tagged *N*-methylglycamines; ultramicro carbohydrate analysis; argon laser.**

Recent advancement of glycobiology has stimulated development of high-performance analysis of carbohydrates. The carbohydrate comprises a vast number of polyhydroxyl compounds including a variety of isomers and homologues, which often coexist with each other. Therefore, carbohydrate analysis requires a method capable of high-resolution separation. Various types of chromatography and electrophoresis have been used for this purpose, and capillary electrophoresis (CE)[3] is currently the most powerful method, since its high capability in separation matches well with this demand.

We published the first paper on CE of carbohydrates in 1989 using 2-aminopyridine-derivatized monosaccharides as models (1). Many other papers (e.g., 2–6, arbitrary choice) as well as a number of reviews (7–16) have hitherto been published on carbohydrate analysis by CE. A tabulated review given out by Suzuki and Honda (16) is especially convenient for literature search on this subject.

Since the publication of the first paper our effort has been focused on the development of a system for routine analysis of carbohydrates by CE. Since carbohydrates generally have neither chromophores nor fluorophores, they should be converted to derivatives detectable by the conventional methods. A number of papers have devoted to this subject, including those utilizing 4-aminobenzoic acid (17); its ethyl ester (3);

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[3] Abbreviations used: CE, capillary electrophoresis; 2-AA, 2-aminoacridone; ANTS, aminonaphthalene; PMP, 1-phenyl-3-methyl-5-pyrazolone; LIF, laser-induced fluorescence; CBQCA, 3-(4-carbobenzoyl)-2-quinolinecarboxaldehyde; APTS, 8-aminopyrene-1,3,6-trisulfonate; NBD-F, 7-nitro-2,1,3-benzoxadiazole; MG, *N*-methylglycamines; PNGase F, glycoamidase from *Flavobacterium meningosepticum;* CE-VIS, CE with detection by absorption in the visible region; NeuAc, *N*-acetylneuraminic acid.

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its nitrile (18); *N*-(4-aminobenzoyl-L-glutamate (19); 6-aminoquinoline (5); 2-aminoacridone (2-AA) (20); isomeric aminonaphthalene mono- (21); di- (22); and tri- (ANTS) (4) sulfonates; 8-aminopyrene 1,3,6-trisulfonate (6), etc. However, all of the reported methods using the above reagents are based on reductive amination which requires acid catalyst. These methods have, therefore, theoretical possibility of releasing acid-labile moieties such as the sialic acid residue and the N- as well as O-sulfate groups. For this reason we proposed a method of a different type, based on the condensation of reducing carbohydrates with 1-phenyl-3-methyl-5-pyrazolone (PMP). The PMP method is suitable for routine analysis, because the reaction is quantitative under mild conditions, the procedure is simple, and the derivatives give strong absorption (ϵ ca. 30,000) in the UV region (λ_{max} 245 nm). In addition, it causes neither desialylation nor desulfation during derivatization. PMP derivatives can be well separated by a number of modes (e.g., 23–29). The detection limit of the derivatives is, however, at the micromolar concentration at best, which corresponds to the femtomole level as injected amount when a 50-μm-i.d. capillary is used. In-capillary preconcentration proved to be effective for lowering the detection limit to as low as the attomole level, but quantification was not reproducible (unpublished results). A much more sensitive method is required for special purposes dealing with extremely minute amounts of samples, especially from biological sources.

One of the promising methods for ultrasensitive detection of carbohydrates separated by CE is the use of laser-induced fluorescence (LIF). Liu and co-workers (2) developed a method for derivatization based on reductive amination with the ammonium salt, followed by condensation with 3-(4-carbobenzoyl)-2-quinolinecarboxaldehyde (CBQCA) in the presence of the cyanide ion leading to isoindole derivatives. The derivatives could be detected at the attomole level in a narrow capillary by fluorescence induced by argon laser. However, the derivatization required a rather time-consuming two-step procedure, and the first step is not quantitative under the conditions employed. Zhang and co-workers (30) reported an alternative method based on coupling of aminated sugars to a succinylimidated rhodamine (TRSE) dye. They prepared the intermediate aminated sugars by reductive benzylamination of aldoses followed by debenzylation, but the details of the analytical procedure were not shown. The tetrabromorhodamine succinyl ester derivatives fluoresce when excited by helium–neon laser. Guttman and co-workers (6) developed a method for LIF detection of separated 8-aminopyrene-1,3,6-trisulfonate (APTS) derivatives of carbohydrates also using argon laser. This method is convenient because the derivatization requires only one step, but is again based on reductive amination which requires acid catalyst. The 2-AA and ANTS derivatives also fluoresce when irradiated by helium–cadmium laser, but the methods utilizing these reagents have the same problem as the APTS method.

Thus, all these reported methods of derivatization for LIF detection in capillary electrophoresis have a common problem of using acid catalyst, which may cause release of the sialic acid residues. In addition some of them are not quantitative and the others must use roundabout synthetic route. We have overcome this problem by replacing reductive amination employed by Liu and co-workers by N-methylamination with methylamine whose nitrogen atom has higher electron density and accordingly higher reactivity than ammonia. We also replaced CBQCA by 7-nitro-2,1,3-benzoxadiazole (NBD-F) for introducing a fluorescence tag to the resultant *N*-methylglycamines (MG), since it can be introduced much more smoothly without using a toxic reagent such as a cyanide salt (2). Thus, we could develop a convenient one-pot method based on reductive N-methylamination, followed by labeling with NBD-F.

This paper describes the details of this NBD–MG method and presents a few applications for the analysis of mono- and oligosaccharides.

MATERIALS AND METHODS

Chemicals. A reagent-grade methylamine (ca. 40 w/v% aqueous solution) was obtained from Kishida Chemicals (Honmachibashi, Chuo-ku, Osaka). NBD-F was purchased from Tokyo Kasei Kogyo (Nihonbashi-honcho, Chuo-ku, Tokyo). Dimethylamine–borane complex was from Sigma Aldrich Japan (Nihonbashi Horidome-machi, Chuo-ku, Tokyo). The analytically pure samples of monosaccharides and the authentic specimen of *N*-methylglucamine were obtained from Wako Pure Chemicals (Dosho-machi, Chuo-ku, Osaka). The sample of calf fetal fetuin was from Sigma Chemical Company (St. Louis, MO). Glycoamidase from *Flavobacterium meningosepticum* (PNGase F) was purchased from Biocarb (Beverly, MA). All other chemicals were of the highest grade commercial available. Methanol and ethanol were of liquid chromatography grade. All the above reagents, solvents, and carbohydrate as well as glycoprotein samples were used as obtained. Deionized and glassware-distilled water was used for preparation of electrophoretic solutions. Before each series of analysis the electrophoretic solution to be used was degassed *in vacuo* under sonication. A membrane filter (Ultrafree C3GC, exclusion limit, 10,000) for ultrafiltration and an octadecyl (PDS) column (OASIS HLB, 3 ml) for cleanup of the products were obtained from Millipore Corporation (Bedford, MA) and Waters Corporation (Milford, MA), respectively.

General procedure for derivatization. A sample of a monosaccharide or a mixture of mono-/oligo-saccharides was dissolved in an aqueous methylamine solution (10 μl) containing dimethylamine–borane complex. The amount of sample, the pH value, and the concentrations of methylamine and dimethylamine borane complex are indicated in each experiment. The solution was maintained at an indicated temperature for a specified period of time. The solution was evaporated to dryness under the nitrogen atmosphere at 60°C, methanol and toluene (100 μl each) were added to the residue, and the mixture was evaporated to dryness. The last operation was repeated twice more. The residue was dried up under reduced pressure in a desiccator containing phosphorus pentoxide. The resultant glycamine derivative or a mixture of glycamine derivatives was dissolved in a borate buffer (5 μl) and an NBD-F solution (5μl) was added. The concentration and pH value of the borate buffer and the NBD-F concentration for NBD-tagging are indicated in each experiment. The solution was kept for a specified period of time at an indicated temperature, and the solution was evaporated to dryness. The residue was dissolved in an appropriate volume of water and the solution was subjected to CE analysis.

Standard procedure for the derivatization for CE with detection by absorption in the visible region (CE-VIS). Based on the optimization studies the standard procedure for CE-VIS was established as follows. Dissolve a sample of a carbohydrate (1 nmol–1μmol) or a mixture of carbohydrates each in the same range, taken in an Eppendorf tube, in an aqueous solution (100 μl) containing methylamine and dimethylaine—borane complex to concentrations of 1 M and 200 mM, respectively, which has been adjusted to pH 4.5 with acetic acid, and incubate the solution for 30 min at 40°C. Evaporate the reaction solution by placing the vessel in an oven thermostated at 60°C and flushing the surface of the solution by the nitrogen gas. Add methanol (100 μl) and toluene (100 μl), evaporate the solution again in a similar manner, and finally dry up the residue in a desiccator containing phosphorus pentoxide. Dissolve the residue of *N*-methylgycamine(s) thus prepared, in 200 mM borate buffer, pH 8.0 (5 μl); add a 300 mM NBD-F solution in ethanol (5 μl); and incubate the mixed solution for 5 min at 70°C. Evaporate the solvent by flushing it by the nitrogen gas and redissolve the residue in water (100 μl). Analyze this analytical sample solution of NBD-tagged *N*-methylglycamine derivatives by CE-VIS under appropriate conditions.

Standard procedure for the derivatization for CE-LIF. The procedure is similar to that in CE-VIS, but lower the range of sample amount to 100 fmol to 100 pmol. Also reduce the concentrations of methylamine, dimethylamine–borane complex, NBD-F, and borate buffer to 100, 20, 3, and 10 mM, respectively, and the final analytical sample solution in water (10 μl).

Release of N-glycans from fetuin. This was carried out by modifying the procedure developed by the carbohydrate analysis group of Applied Biosystems (presently Perkin–Elmer). A sample of fetuin (10 μg) was dissolved in a 10% mercaptoethanol solution (10 μl) containing sodium dodecyl sulfate (5%) and the solution was heated on a boiling water bath for 10 min. To the denatured protein were added 500 mM sodium phosphate, pH 7.5 (10 μl), 10 w/v% NP-40 (1 μl), and PNGase F (500 U/μl, 2 μl) and the mixture was incubated for 18 h at 37°C. After heating the mixture on a boiling water bath for 5 min to inactivate the enzyme, it was evaporated to dryness. The residue was subjected to derivatization for CE-LIF by the standard procedure described above.

Cleanup experiments. Seven batches of fetuin sample (10 μg each) were sequentially digested with PNGase F and derivatized according to the procedure described above. Each of the finally obtained residues was dissolved in water (10 μl), and the solution was subjected to one of the following cleanup operations: ethanol precipitation (addition of 20 vol of ethanol and centrifugation), ultrafiltration through a membrane filter, passage through an ODS column followed by washing with water, and extraction with either ethyl acetate, chloroform, or 1-butanol. In the extraction methods each of the solution was extracted three times with the same volume of either solvent.

CE. A preliminary study of CE-VIS was carried out using a Model HEL30P high voltage power supply of Matsusada Precision Devices (Kusatsu, Shiga, Japan) and an 870-CE-VIS detector of JASCO (Hachioji, Tokyo) equipped with a tungsten lamp and an 833-A data processor of Hitachi (Toranomon, Minato-ku, Tokyo). A sample solution was loaded into a capillary by maintaining, for 10 s, the sample solution level at the capillary inlet 10 cm higher than the cathodic solution level.

The major part of the experiments of CE-LIF was performed with a Beckman P/ACE 2000 apparatus equipped with a high-voltage power supply, a pressure injector, an LIF detector with an argon laser (3 mW, 488 nm), and a data processor. Other part of CE-LIF experiments was performed with a Bio-Rad BioFocus 3000 apparatus, which is made up by similar parts including an LIF detector with a 3-mW (488-nm) argon laser. An optical device in this detector was specially designed to obtain the maximal fluorescence intensity.

A roll of a 50-μm-i.d. fused silica tube was obtained from Polymicro Technologies (Phoenix, AZ). A 50 × 60-cm piece was cut out from the role and installed on each of the above apparatus after a window was cre-

ated at a ca. 10-cm position from the outlet by burning the polyimide coating with a small flame. In an experiment aiming at obtaining the highest sensitivity a 50-cm piece of a 10-μm-i.d. fused silica tube obtained from the same source was similarly fitted to the Bio-Focus 3000 apparatus. After the tube was installed on each apparatus, it was flushed with 100 mM sodium hydroxide for 10 min and then rinsed with water. Before each run the tube was equilibrated with the running buffer for 10 min.

Alditol determination by HPLC with postcolumn derivatization. This was carried out by modifying the procedure previously reported by Honda and co-workers (31). Briefly as follows, a reaction product of reductive N-methylamination was applied to a jacketed stainless-steel column (8 mm i.d., 8 cm long) packed with Hitachi 2633 resin (particle diameter, 11 μm), an anion-exchanger having the quaternary ammonium group, which was equilibrated with 500 mM borate buffer (pH 7.1) and thermostated at 65°C by circulating hot water through the jacket, and the column was eluted with the same buffer at a flow rate of 500 μl/min. To the eluate was added a 50 mM solution of sodium metaperiodate supplied by a reagent pump through a Teflon T-joint at a flow rate of 500 μl/min. Periodate oxidation occurred while the mixed effluent was flowing through a Teflon tube (200 μm i.d., 10 m). Subsequently a 15% ammonium acetate solution containing 2,4-pentanedione (2%) and sodium thiosulfate (200 mM) was added to the mixed effluent through another T-joint using a different reagent pump, and the final mixed effluent was forced into a Teflon tube of the same dimension immersed in a silicone oil bath thermostated at 100 $\pm$ 1°C. The formaldehyde formed by periodate oxidation of alditol (glucitol in this case) was converted to the fluorescent dehydropyridine derivatives by the Hantzsche reaction. After cooling the reaction mixture by further flowing it through a 2-m Teflon tube immersed in a water bath, it was led into a Hitachi 650 fluorescence detector. The products from the alditol was monitored at the excitation and emission wavelengths of 410 and 503 nm, respectively. Under the conditions employed the authentic *N*-methylglucamine and glucitol eluted at 35 and 42 min, respectively,

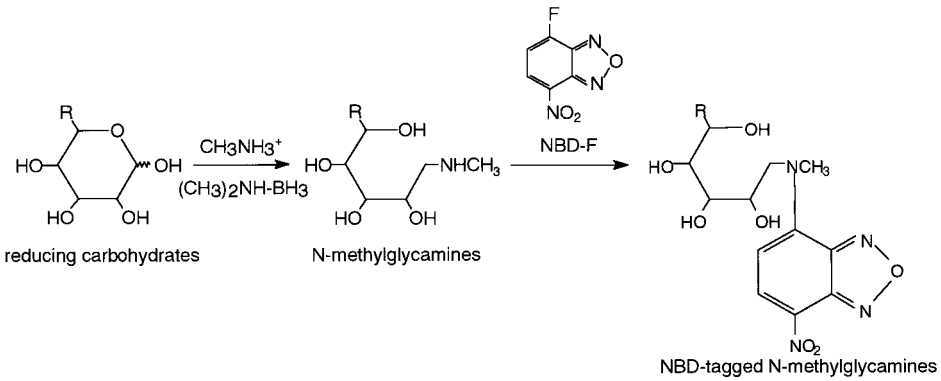
RESULTS AND DISCUSSION

Strategy for Developing a Derivatization Method for CE-LIF

LIF allows ultrasensitive detection and the use of argon laser is especially promising, because its 488-nm beam gives high-energy density on the detection window, and the interference by surroundings is small because sample matrix generally does not emit at such a long wavelength as that of argon laser. Since intact carbohydrates do not fluoresce, they must be converted to derivatives excitable at this wavelength and fluorescence-emissive at a wavelength sufficiently far from the excitation wavelength.

Among various derivatives that meet these requirements, NBD derivatives are promising, because the derivatization using NBD-F takes place very smoothly and the yields are quite high. In order to use this derivatization method a carbohydrate sample must have either the amino or imino function. The earlier work of Imai's group (32, 33) indicated that imino acids are more reactive to NBD-F than amino acids, presumably due to higher electron density of the nitrogen atom. Therefore, introduction of an alkylamino group to a reducing carbohydrate is considered to be beneficial for tagging it with NBD, and this modification will be possible through the N-alkylglycosylation of the reducing end. However, this type of reaction is reversible and will regenerate the starting material giving low yield of *N*-alkylglycosylamine and in addition it may have anomeric diversity. Reductive N-alkylamination leading to *N*-alkylglycamines, via the Schiff bases, is considered to be much better from this viewpoint. Therefore, we optimized reductive N-methylamination, as it is the simplest and the most promising reaction for the first step of the proposed method.

Conversion of reducing carbohydrates to *N*-methylglycamines requires methylamine and an appropriate reductant. The former is commercially available as its aqueous solution. Various reductants working in aqueous solutions are available, including sodium borohydride, sodium cyanoborohydride, diborane, dimethylamine–borane complex, etc. Our preliminary examination using glucose as a model carbohydrate indicated that the reducibility of sodium borohydride toward the Schiff base was not sufficient for quantitative derivatization and it concurrently reduced the starting carbohydrates to glucitol to a considerable extent. Sodium cyanoborohydride yielded the cyanide ion in the reaction solution, which interfered with the condensation of the resultant *N*-methylglucamine with NBD-F for unknown reason. In contrast to these reductants, dimethylamine–borane complex reacted smoothly with the Schiff base to give *N*-methylglycamines in high yields. HPLC with postcolumn periodate oxidation followed by the Hantzsche reaction (31) under the conditions which allow separation of the authentic *N*-methylglucamine and glucitol actually gave only the *N*-methylglucamine peak but no trace of the glucitol peak (data not shown). This is an evidence indicating that there was no side reaction leading to glucitol with this reductant. It is also notable that the excess amount of this reductant could easily be removed from the reaction solution after reduction together with the solvent, simply by evaporation.



SCHEME 1. Derivatization of reducing carbohydrates to the NBD-tagged *N*-methylglycamines.

From the foregoing reasons we adopted a strategy of reductive N-methylamination with dimethylamine–borane complex, followed by introduction of a NBD tag to the resultant *N*-methylglycamine (Scheme 1).

Optimization of the Derivatization Conditions for CE-VIS

Based on the above considerations we undertook optimization studies to derivatize reducing carbohydrates to NBD-tagged *N*-methylglycamines tentatively with photometric monitoring at 490 nm using a 10 mM glucose solution as a model. The volume of the solution of the finally obtained product was adjusted to 100 μl. In each optimization study only the varied factors are described. The yield of *N*-methylglucamine was estimated by comparing the peak area of the final product with that from the authentic specimen of *N*-methylglucamine.

Reductive N-methylamination (first step). First we examined whether the dimethylamine–borane complex should be added after the Schiff base has been formed or simultaneously with methylamine. It was shown that the yields of *N*-methylglucamine in sequential and simultaneous additions were 101 and 99%, respectively. Thus, the yields were quantitative and there was no substantial difference between the two addition modes, though the former mode gave a slightly higher yield. Based on this result we performed optimization study by simultaneous addition of methylamine and dimethlyamine–borane complex for the sake of simplicity.

Since the concentration of methylamine should be as high as possible to facilitate *N*-methylglucamine formation because reaction rate is generally proportional to reactant concentration, it was fixed to 1 M, practically the highest concentration attainable. High concentrations of dimethylamine–borane complex will also be beneficial for reductive N-methylamination. An optimization experiment indicated that more than 20-fold concentration (200 mM) to glucose was necessary for complete reaction, demonstrating this inference (figure not shown). Under the above conditions the molar ratio of methylamine to dimethyamine–borane complex was 5.

Next, pH dependence of the yield of *N*-methylglucamine was examined, changing the pH value by addition of acetic acid. The result indicated that the yield of *N*-methylglucamine was almost quantitative below pH 4.5 (data not shown). Since pH should be as high as possible in order to suppress release of the sialic acid residue from carbohydrate samples, a value of 4.5 was adopted in the later experiments.

Under the conditions of 1 M methylamine, 200 mM dimethylamine–borane complex, pH 4.5, and reaction time of 30 min, the yield of *N*-methylglucamine increased rapidly as temperature rose to 20°C and stayed at the maximum value until 40°C. However, it decreased above 40°C, because the starting carbohydrate (glucose) was also directly reduced to glucitol at higher temperatures, as evidenced by the presence of the glucitol peak on HPLC with postcolumn derivatization (the yield of glucitol, ca. 10% at 60°).

The yield of *N*-methylglucamine rapidly increased and a plateau of ca. 100% was reached in 30 min with 1 M methylamine, 200 mM dimethylamine–borane complex, pH 4.5, at 40°C and stayed at this value at least until 100 min (figure not shown).

Based on these results the optimum conditions for reductive N-methylamination for CE-VIS were methylamine, 1 M; dimethylamine–borane complex, 200 mM; pH, 4.5 with acetic acid; reaction temperature, 40°C; and reaction time, 30 min for 10 mM glucose. One of the advantages of the proposed MG–NBD method is such mildness of reaction conditions, due to higher basicity of methylamine than ammonia. Reductive amination used in the CBQCA method (2) requires much more drastic conditions (100°C, 100–120 min). In the APTS method (6) tetrahydrofuran containing a

high concentration (15%) of acetic acid is used as reaction medium and a reaction time of 1–2 h is required at 55°C. The higher temperature causes considerable degree of desialylation due to the use of the acid as catalyst.

Coupling to NBD-F (second step). The labeling of amines/imines with NBD-F has been thoroughly investigated by the group of Imai (e.g., 32, 33), and suitable reaction conditions have been established. Reagent solution of NBD-F can be prepared in water-miscible solvents such as ethanol and acetonitrile. Use of alkaline borate buffer as reaction medium facilitates the condensation of the amino/imino groups with NBD-F, and this medium is also profitable for separation of carbohydrates by zone electrophoresis as borate complexes.

The excess reagents and the solvent in the reaction mixture of reductive N-methylamination could be removed by coevaporation of the reaction solution with toluene and methanol under the nitrogen atmosphere. The *N*-methylglucamine resulting from 100 mM glucose was allowed to react with NBD-F by dissolving the residue in borate buffer, adding an NBD-F solution, and incubating the resultant solution for a short period of time. A 300 mM solution of NBD-F in ethanol resulted in satisfactory tagging for CE-VIS. Analysis of the final product indicated that 5 min was enough for quantitative tagging at 70°C. Such rapidity of the condensation is also comparable to the formation of the isoindole derivatives from glycamines in the CBQCA method (1 h) (2).

A Typical Example of the Analysis of Reducing Carbohydrates by CE-VIS

A mixture of monosaccharides (1 μmol; each) commonly found in glycoproteins was subjected to the derivatization by the standard procedure for CE-VIS, and the product was analyzed by CE-VIS. The reason why we did not use free hexosamines but included their *N*-acetates is because the presence of the free amino group at C-2 hampers reductive N-methylamination to give low yields of derivatives. In the monosaccharide composition analysis of glycoproteins the monosaccharides must be released by acid hydrolysis. During hydrolysis the *N*-acetyl group is removed to give free amino acids, but they can easily be re-N-acetylated by stirring with acetic anhydride in the presence of pyridine. Figure 1 shows an example separated by zone electrophoresis mode as borate complexes using 200 mM borate buffer, pH 10.5, containing methanol to a concentration of 10% (v/v) as electrophoretic solution. Although the addition of methanol caused retardation of all peaks, it was beneficial for improvement of separation. Since the final product was dissolved in 100 μl of water for the preparation of analytical sample solution, the concentration of the product analyzed was equivalent to 100 mM each.

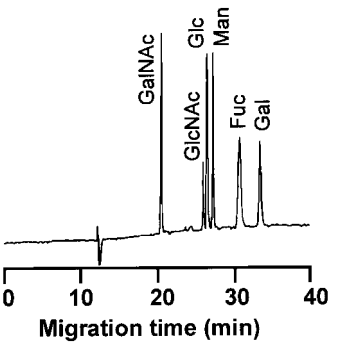


FIG. 1. Analysis of the monosaccharides commonly found in glycoproteins by CE-VIS as NBD-tagged *N*-methylglycamines. A mixture of the monosaccharides (1 μmol each) was derivatized by the standard procedure for CE-VIS. Analytical conditions: capillary, fused silica (50 μm i.d., 70 cm); electrophoretic solution, 200 mM borate buffer, pH 10.5, containing methanol (10%, v/v); applied voltage, 15 kV; detection, absorption at 490 nm. Peaks are of the NBD-tagged *N*-methylglycamine derivatives of *N*-acetylgalatosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), glucose (Glc), mannose (Man), fucose (Fuc), and galactose (Gal).

All these monosaccharides were well separated from each other under these conditions and gave linear calibration graphs at least over a concentration range of 100 μM–100 mM of the final analytical sample solution (100 fmol–1 nmol range as injected amount), using glycine as internal standard. The coefficients of correlation were in a range of 0.995–1.000, and the relative standard deviation ($n = 7$) at the 5 mM level was less than 3.5%. The molar response varied among monosaccharides to a considerable extent for unknown reasons. The detection limit was at the 1 μM level. Thus, CE-VIS of NBD-tagged *N*-methylglucamine derivatives allowed rapid, practical analysis of reducing carbohydrates.

Further Optimization for CE-LIF

CE-LIF generally allows analysis of reducing carbohydrates of down to the attomole to femtomole amounts. Such high sensitive analysis is liable to be affected by a number of factors arising from sample matrix and hardware. The foregoing result obtained for CE-VIS indicated that the optimum concentrations of methylamine and dimethylamine–borane complex were as high as 1 M and 200 mM, respectively. However, in CE-LIF using much lower sample concentrations such high concentrations of methylamine and dimethylamine–borane complex will not be required. Therefore, they were reoptimized for CE-LIF using an equimolar (10 μM) mixture of monosaccharides in 100 μl of reaction solution. Unexpectedly, however, baseline was not noisy and there was no drift for any

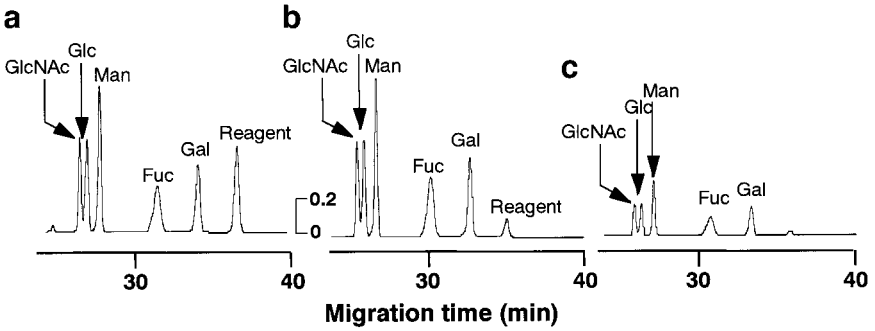


FIG. 2. Effect of methylamine concentration on the first-step reaction (reductive N-methylamination) of monosaccharides for CE-LIF. Methylamine, 1 M (a), 100 mM (b), and 10 mM (c). Monosaccharide, 100 pmol each; dimethylamine–borane complex, 20 mM; volume of reaction solution, 100 μl; pH, 4.5; reaction temperature, 40°C; reaction time, 30 min. NBD tagging was performed under the following conditions. Reagent solution: 3 mM NBD-F in ethanol (5 μl); reaction medium, 10 mM borate buffer, pH 8.0 (5 μl); reaction temperature, 70°C; reaction time, 5 min. The final product was dissolved in water (10 μl) to make up an analytical solution. Therefore, the NBD-tagged *N*-methylglycamines in the analytical sample solution were at a concentration of 10 μM. Analytical conditions for CE-LIF: capillary, fused silica (50 μm i. d., 57 cm); electrophoretic solution, 200 mM borate buffer, pH 10.5; applied voltage, 15 kV; detection, argon laser-induced fluorescence (520 nm).

methylamine concentrations tested, i.e., 10 mM, 100 mM, and 1 M (Fig. 2).

Ten millimolar methylamine gave significantly smaller peaks of the NBD-tagged *N*-methylglycamines than 100 mM methylamine, while the peak intensity was not different between 100 mM and 1 M. However, the relative intensity of the reagent peak at ca. 36 min for 1 M methylamine was much larger than that for 100 mM methylamine.

These results indicate that the methylamine concentration can be lowered to approximately one order of magnitude from that in CE-VIS. Since sample concentration in the reaction solution of the first-step reaction for CE-LIF is usually lower than 1 μM which was used in this reoptimization study, the 100 mM concentration will generally be enough for CE-LIF. The molar concentration ratio of dimethylamine–borane complex to methylamine was rather arbitrarily maintained at one-fifth (20 mM). The other conditions for reductive N-methylamination were also not altered. The other conditions for derivatization and product analysis are indicated in the legend to Fig. 2.

The concentration and pH of the borate buffer used as the medium for NBD tagging were also reexamined, because these factors are expected to give considerable influence on the yield of the NBD-tagged *N*-methylglycamines and baseline drift. The results in Fig. 3 show that the high borate concentrations as used for CE-VIS were rather disadvantageous, whereas the pH dependence was not different from that in CE-VIS.

Figure 4 shows the influence of the NBD-F concentration on tagging. At such a high concentration of 300

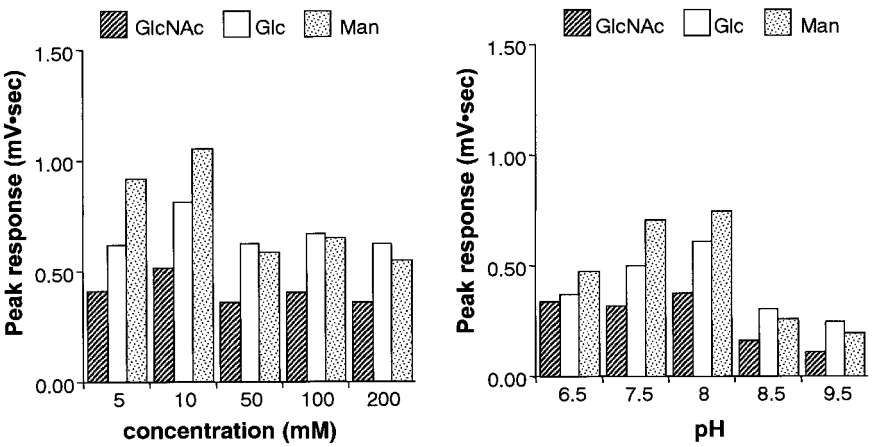


FIG. 3. Effects of borate concentration and pH of the reaction medium on the NBD-tagging of *N*-methylglycamines for CE-LIF. The borate concentration and pH were varied between 5 and 100 mM and between 6.5 and 9.5, respectively. Methylamine, 100 mM. The other reaction conditions as in Fig. 2. Analytical conditions also as in Fig. 2. Bars are of the NBD-tagged *N*-methylglycamine derivatives of *N*-acetylglucosamine (▨), glucose (□), and mannose (▩).

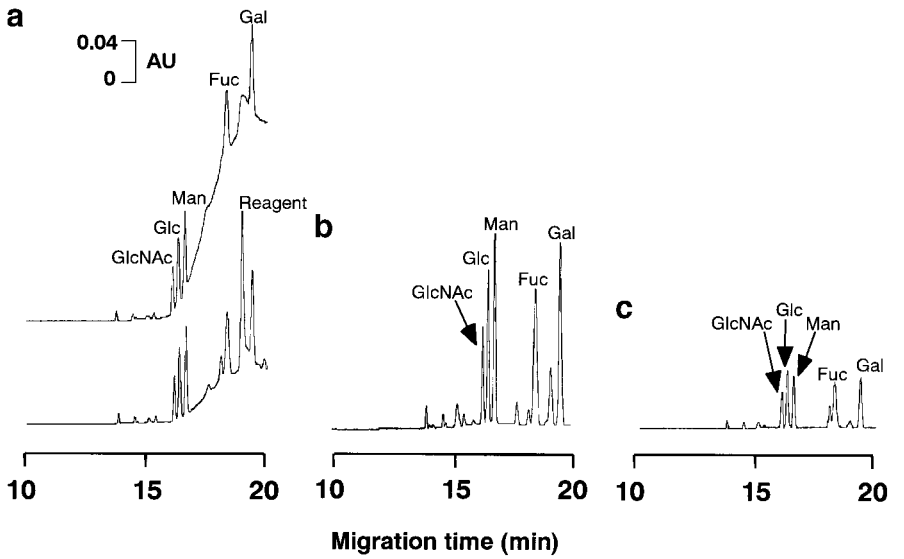


FIG. 4. Effect of the NBD-F concentration on the NBD tagging of *N*-methylglycamines for CE-LIF. NBD-F, 30 mM (a), 3 mM (b), and 0.3 mM (c) in ethanol. Methylamine, 100 mM. The other reaction conditions as in Fig. 2. Electrophoretic solution, 200 mM borate buffer, pH 10.0, containing SDS to a concentration of 50 mM. Analytical conditions as in Fig. 2.

mM as used for CE-VIS monosaccharide analysis was seriously disturbed by rapid baseline raising after 15 min, due to the interference by the sample matrix. The interference was observed even at 30 mM. At 0.3 mM, however, peak intensity became low due to incomplete reaction. After all, the 3 mM concentration was optimal, though a few minor peaks due to the reagent blank were still observed.

Thus, it was shown that the concentrations of methylamine and borate buffer should be reduced to one order of magnitude. Since the NBD-F concentration gave larger influence on the baseline, it should be reduced by two orders of magnitude. The resultant NBD-tagged *N*-methylglucamine fluoresced with excitation and emission maxima at ca. 470 and 520 nm, respectively. Although the wavelength of the excitation maximum was slightly deviated from the wavelength of argon laser, i.e., 488 nm, the fluorescence at ca. 520 nm was sufficiently intense to allow sensitive detection of these derivatives.

Stability of the Sialic Acid Residue

The mildness of reaction conditions brings forth an advantage that it does not cause desialylation from sialylated glycans. Figure 5 demonstrates the stability of the sialic acid residue in 6′-*N*-acetylneutraminyllactose as a model during derivatization, based on the analysis of the NBD-tagged *N*-methylglycamine derivatives by CE-LIF.

If the sialic acid residue were released during derivatization, a peak of NBD-tagged *N*-methyllactamine should be detected in the electropherogram at ca. 34 min (Fig. 5, arrow). The result indicated a large peak of NBD-tagged *N*-methyl-6′-*N*-acetylneuraminyllactamine at ca. 38 min together with a small peak of NBD-tagged *N*-methyllactamine. However, this peak was very small and the amount of lactose estimated from this minor peak was almost the same as that in the 6′-*N*-acetylneuraminyllactose sample as an impu-

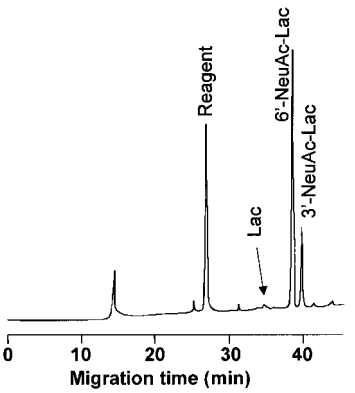


FIG. 5. Stability of the 6′-*N*-acetylneuraminyllactose during derivatization. A commercial sample of 6′-*N*-acetylneuraminyllactose was derivatized by the standard procedure for CE-LIF. Electrophoretic solution, 200 mM borate buffer, pH 10.5, containing methanol to a concentration of 10% (by volume); applied voltage, 10 kV. The other analytical conditions as in Fig. 2. The peak at ca. 34 min is indicative of the derivatized lactose contained in the sample as an impurity but not the derivative of lactose resulting from partial hydrolysis. Peaks are of the reagent blank (Reagent) and the NBD-tagged *N*-methylglycamine derivatives of lactose (Lac), 6′-*N*-aecty-neuraminyllacose (6′-NeuAc-Lac), and 3′-*N*-acetylneuraminyllactose (3′-NeuAc-Lac).

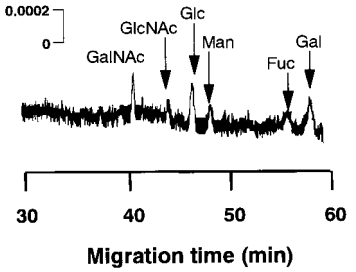


FIG. 6. Analysis of the monosaccharides commonly found in glycoproteins by CE-LIF as the NBD-tagged *N*-methylglycamine derivatives at the 100 nM level for the analytical solution (1 fmol level as injected amount). The derivatization was performed by the standard procedure for CE-LIF. Electrophoretic solution, 200 mM borate buffer, pH 10.5, containing SDS (200 mM). The other analytical conditions as in Fig. 2. Peaks assigned as in Fig. 1.

rity. This result clearly demonstrates the high stability of the sialic acid residue in this series of derivatization.

Application of CE-LIF to the Analysis of Mono- and Oligosaccharides in Glycoproteins

A mixture of monosaccharides commonly found in glycoproteins, the same species as used for CE-VIS, were analyzed by CE-LIF under the established conditions. Figure 6 exhibits an example obtained from a 1-pmol mixture.

The analytical sample solution was prepared in 10 μl; hence the concentration of each derivative was equivalent to 100 nM. The injected amount was at the 1-fmol level, using a 50-μm-i.d. capillary. Linear calibration curves were obtained at least over a range of 100 nM–50 μM for analytical sample solution. Analysis of monosaccharide derivatives of down to 10 amol injected amount was possible, when a 10-μm-i.d. tube was used.

Oligosaccharides could also be analyzed by CE-LIF as NBD-tagged *N*-methylglycamines. Although a number of separation modes are possible similarly to PMP derivatives (23–29), we present here an examples of oligosaccharide analysis by zone electrophoresis as borate complexes. Figure 7 shows simultaneous analysis of dextran oligomers at the 1 μM level (for analytical sample solution) using a 50-μm-i.d. tube. The inset shows an expanded electropherogram for the d.p. 4–13 oligomers.

Application to analysis of glycoprotein *N*-glycans was also investigated. In this case the *N*-glycans must be released from the polypeptide core prior to analysis. Of the two available methods based on chemical reaction (hydrazinolysis) and enzymatic digestion (digestion by glycoamidase from *F. menigosepticum*, PNGase F), the former have no specificity to carbohydrate structure and gives cleaner product, since the excess reagent can be removed by evaporation. Nevertheless, we adopted the latter method using PNGase F, because we considered the safety of the sialic acid residues is more important.

A 10-μg amount of fetuin as a model protein was digested with PNGase F under the optimized conditions and the digest was evaporated to dryness. The residue was reductively N-methylaminated and reacted with NBD-F by the standard procedure for CE-LIF. Since direct analysis of the final product gave desperate result with the target peaks of the derivatives of *N*-glycans buried under huge blank peaks, various methods of cleanup were tested for the final product and results were compared. Figures 8a–8c show the effects of cleanup by ethanol precipitation, ultrafiltration through a membrane filter, and passage through a small ODS column, respectively.

Under the employed analytical conditions of micellar electrokinetic chromatography mode the peaks of the NBD-tagged *N*-methylglycamine derivatives of *N*-glycans appeared at around 10 min, but comparison with blank experiments (top charts) indicated that they were followed by large peaks due to the reagent and the digestion/derivatization matrices. In the cleanup methods by ultrafiltration (Fig. 8b) and passage through an ODS column (Fig. 8c) the target *N*-glycan derivatives themselves were interfered by minor blank peaks. In contrast to these methods solvent extraction showed much more efficient removal of interfering peaks (Fig. 9).

The number of blank peaks was generally fewer. In the extraction with ethyl acetate (Fig. 9a) there were no peaks overlapping with NBD-tagged *N*-methylglycamine peaks, and the large peak at ca. 17 min and minor peaks in a range of 12–16 min did not give significant influence on the analysis of *N*-glycans.

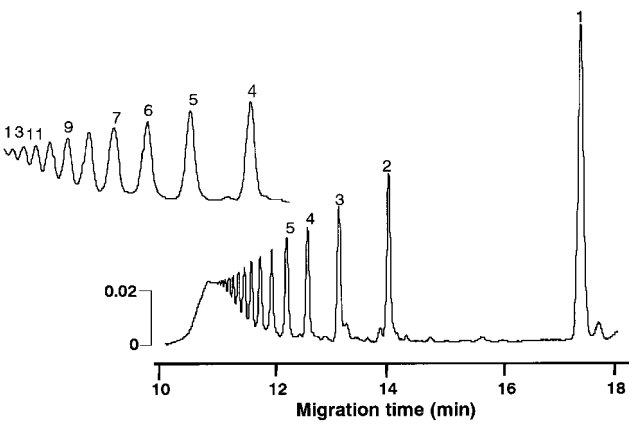


FIG. 7. Analysis of dextran oligomers by CE-LIF as NBD-tagged *N*-methylglycamines at the 1 μM level for the analytical solution. The derivatization was performed by the standard procedure for CE-LIF. Electrophoretic solution, 50 mM borate buffer, pH 10.0. The other analytical conditions as in Fig. 2.

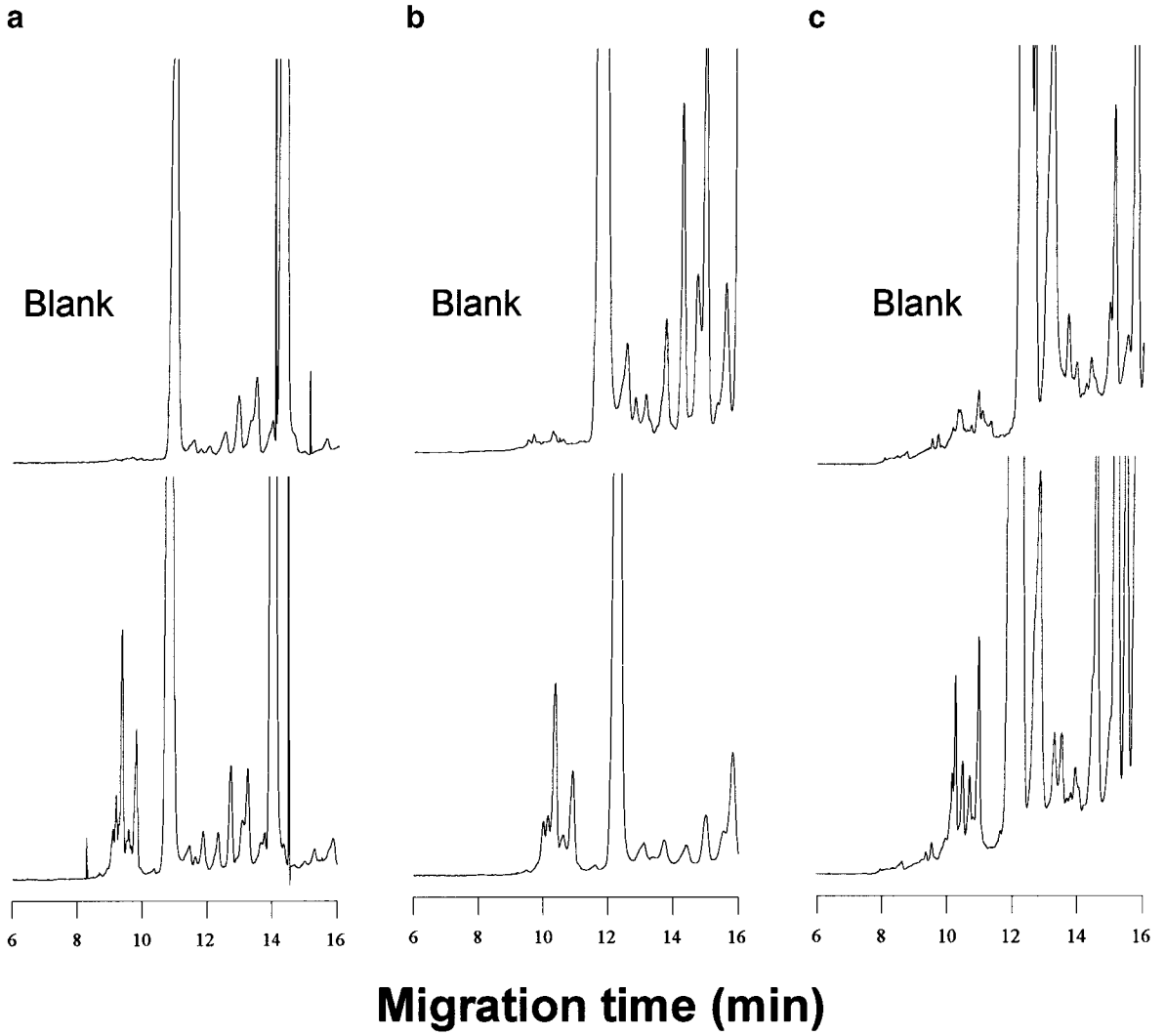


FIG. 8. Effects of cleanup by ethanol precipitation (a), ultrafiltration (b), and passage through an ODS column (c), on the analysis of fetuin *N*-glycans. Fetuin sample (10μg) was digested with PNGase F and the resultant *N*-glycan mixture was reductively N-methylaminated and subsequently reacted with NBD-F by the standard procedures for CE-LIF. The final product was subjected to cleanup by one of the indicated methods. Capillary, fused silica (50 μm i.d., 50 cm); electrophoretic solution, 50 mM borate buffer, pH 6.5, containing SDS (100 mM); applied voltage, 13 kV. The other analytical conditions as in Fig. 2.

Since this solvent was not so strongly polar, loss of the derivatives of *N*-glycans by extraction was negligible.

Figure 10 shows an example of the analysis of fetuin *N*-glycans as NBD-tagged *N*-methylglycamines after cleanup by extraction with ethyl acetate. In this analysis the analytical conditions were further optimized by changing the phosphate buffer to a borate buffer with a higher pH value.

In our previous work (34) we analyzed, as AP derivatives, the desialylated glycan mixture from calf fetuin from the same source by two separation modes (zone electrophoresis in 100 mM phosphate buffer, pH 2.5, and zone electrophoresis as borate complexes in 200 mM borate buffer, pH 10.5) by CE. In both modes the presence of three major *N*-glycans, one nonasaccharide, Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2Manα1 $\rightarrow$ 3(Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2-Manα1 $\rightarrow$ 6)Manβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4GlcNAc, and two positional isomers of undecasaccharides, Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2Manα1 $\rightarrow$ 6[Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2(Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4)Manα1 $\rightarrow$ 3]Manβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4GlcNAc and Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2Manα1 $\rightarrow$ 6[Galβ1 $\rightarrow$ 3GlcNAcβ1 $\rightarrow$ 2(Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4)Manα1 $\rightarrow$ 3]Manβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4GlcNAc, was ascertained. The nondesialylated *N*-glycans in fetuin is known to contain the *N*-acetylneuraminic acid (NeuAc) residues attached to the nonreducing terminal galactose (Gal) residues, but there are variations depending on the degree of substitution by the NeuAc residue and the attaching position. Generally it is not easy to separate and assign

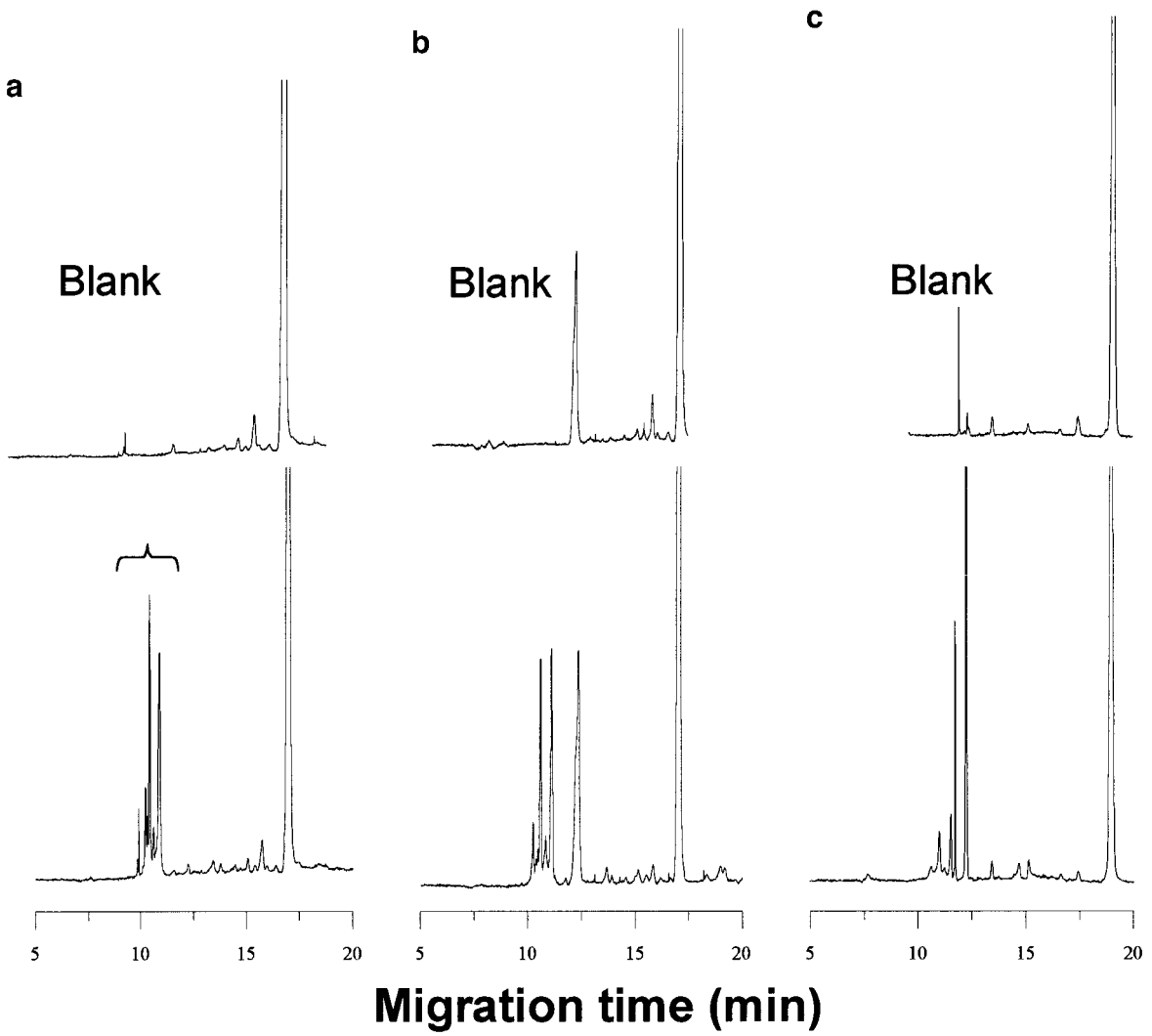


FIG. 9. Effects of the extraction with ethyl acetate (a), chloroform (b), and 1-butanol (c) of the final derivatization mixture on the analysis of fetuin *N*-glycans. Fetuin was treated as in Fig. 8 and the final product was extracted as indicated. Analytical conditions as in Fig. 8. The bracketed peaks were further separated under improved conditions (see Fig. 10).

nondesialylated *N*-glycans, especially as a brand-new type of derivatives. Recently, however, we could analyze them as the PMP derivatives using a running buffer similar to that used in the present work and tentatively assigned the peaks, based on a parallel experiment to isolate individual PMP derivatives of *N*-glycans by preparative liquid chromatography, conversion of the isolated PMP derivatives to the corresponding AP derivatives by our newly developed procedure after checking their peak positions in CE under the conditions used for Fig. 10, and identification by comparison with the authentic specimens. The electropherogram was quite similar to that in Fig. 10 with respect to relative peak intensities of individual peaks and elution order, suggesting that the same separation mechanism exerted between the PMP derivatives and the NBD-tagged *N*-glycamine derivatives. The peak assignment mentioned above is rather roundabout, but a better method will not be available. Anyway, since the procedure for the release of *N*-glycans from fetuin by PNGase F has been well established, the PNGase F digest can be used as a good model of *N*-glycans and can be regarded as the authentic mixture of *N*-glycans. The result in Fig. 10 clearly demonstrates the validity of the proposed method also for the analysis of glycoprotein glycans. The details of the assignment of sialo-*N*-glycan peaks will be published elsewhere in the near future.

CONCLUSION

In this paper a new method is proposed for the derivatization of reducing carbohydrates based on reductive *N*-methylamination of a reducing carbohydrate

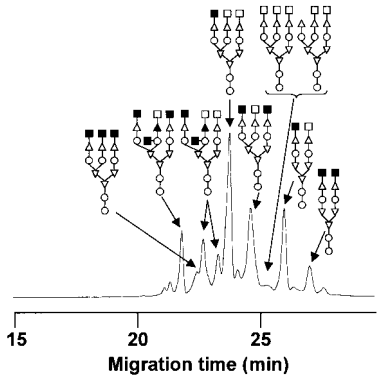


FIG. 10. Analysis of the *N*-glycans from calf fetal fetuin by CE-LIF. The *N*-glycans were released from fetuin by digestion with PNGase F. The digest was derivatized by the standard procedure for CE-LIF, and the accompanying substances were removed by extraction with ethyl acetate. Peaks are tentatively assigned to the structures given above. Electrophoretic solution, 200 mM borate buffer, pH 10.8, containing SDS (100 mM). The other analytical conditions as in Fig. 9. Symbols of monosaccharide unit: □, α2,3-linked *N*-acetylneuraminic acid; ■, α2,6-linked *N*-acetylneuraminic acid; ▲, β1,3-linked galactose; △,β1, 4-linked galactose; ○, β-linked *N*-acetylglucosamine; ▽, α-linked mannose.

followed by introduction of an NBD tag to the resultant *N*-methylglycamine. Although the first step is a kind of reductive amination, the modification of ammonia by the methyl group allowed easier reaction owing to the increased electron density on the nitrogen atom. As a result the reaction proceeds quantitatively under mild conditions of pH 4.5 at 40°C. It was complete in 30 min under the optimized conditions. The subsequent step is also mild and rapid, and the resultant derivatives of NBD-tagged *N*-methylglycamines are sensitively detected by fluorescence with irradiation by argon laser. Although this method involves two-step reactions, it can be carried out in a one-pot fashion. On the basis of extensive optimization studies the standard procedures for CE-VIS and CE-LIF are proposed under Materials and Methods. Because of the mildness of reaction conditions the sialic acid residues are stable during derivatization. This is quite important in mapping glycoprotein glycans, because most of them have sialic acid residues which are considered to have important function in recognition of specific proteins. Release, even though partial release, of this functional group during glycan analysis has been a difficult problem, because it may lead to misunderstanding of its function. Thus, this advantage of the proposed method should be highly evaluated.

The high sensitivity of detection promises application of this method to glycans in small amounts of important biological samples. Unfortunately, however, high sensitive analysis is always destined to suffer from heavy interference by surroundings. The proposed method cannot be an exception, as is obvious from the overlap of the glycan peaks by a number of accompanying substances, when such samples are directly analyzed without cleanup. Our elaborate studies on various cleanup methods gave a solution to this problem. It is ironic that the simplest method of extraction with ethyl acetate gave the most efficient removal of the interfering substances. The example of the analysis of fetuin *N*-glycans at the microgram level with this simple cleanup clearly demonstrated the usefulness of the proposed method. Applications to a wide range of *N*-glycans will appear elsewhere.

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