Focussing Review

Analysis of Carbohydrates by Capillary Electrochromatography

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Abstract

In this review we summarize the recent advances in capillary electrochromatography of carbohydrates, glycoconjugates, and carbohydrate-related compounds. Intact carbohydrates could be separated on ODS columns with aqueous acetonitrile as eluent. However, detection by UV absorption in the ordinary wavelengths did not give enough sensitivity for the detection of separated carbohydrates. Absorption at 195 nm allowed much more sensitive detection, but selectivity was rather problematic. Although the use of a light scattering detector was also possible, it had a similar limitation. Derivatization of carbohydrates enhanced detection sensitivity. Derivatives formed with 1-phenyl-3-methyl-5-pyrazolone could be separated on ODS columns with aqueous acetonitrile and sensitively detected by UV absorption. *p*-Nitrophenyl glycosides of mono- and oligosaccharides were also separated by this system. It was essential that a considerable proportion of silanol groups remained unmodified in the stationary phase in order to induce appropriate velocity of electroosmotic flow for driving the separated solutes in reasonable analysis time. However, the remaining silanol groups caused peak broadening. Tailor-made continuous bed of polyacrylamide columns gave excellent separation, since they were free from this "remaining silanol" problem. Some applications to glycoconjugates are also overviewed to evaluate CEC in biomedical analysis.

Keywords: capillary electrochromatography, high-performance liquid chromatography, carbohydrates, ODS columns, aminosilica columns, electroosmotic flow, in-capillary derivatization, PMP sugars, p-nitrophenylgylcosides

1. Introduction

Carbohydrates are considered to have many key biological functions [1] and have attracted attention of glycobiologists as the most important postscriptomes. When conjugated with proteins to form glycoproteins, they can alter the three-dimensional structure and thereby the function of proteins. As the components of glyco lipids, they can play pivotal roles in cell-cell adhesion and recognition. The extracellular matrix contains proteoglycans, large glycoconjugates that take part in many recognition processes and maintenance of homeostasis.

Although numerous carbohydrate structures occur in nature, only a little is known about the details on the functions of carbohydrates. This can be attributed mainly to the difficulty in their analysis to effect sufficient information. The chromatographic separation of carbohydrates has often met a difficulty in resolution due to the diversity of homologues, coming from the difference of composition, sequence as well as branching, and attaching position as well as mode, of the unit monosaccharides. Strong hydrophilicity is another reason to make separation of carbohydrates difficult. Functional groups, such as the acetamino, *O*- as well as *N*-sulfate, and phosphate groups, give additional variation and hamper easy separation. Thus, the carbohydrate structure is more complex than nucleic acid and protein structures, and needs separation modes with higher resolution. Moreover, the inherent lack of chromophoric/fluorescent function requires conversion of carbohydrates to derivatives amenable to conventional detections. From these reasons, capillary electrochromatography (CEC) of carbohydrates must overcome a number of problems and therefore, only limited numbers of papers have hitherto appeared on this subject (included in a few CEC reviews, [2, 3]).

2. HPLC as the basis of CEC

In these two decades various separation modes and detection methods have been developed for high-performance liquid chromatography (HPLC) of carbohydrates (reviewed for example in Ref [4]). Similarly as in other fields, reverse phase partition chromatog-

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Table 1. Separation modes and systems used for HPLC of intact carbohydrates.

Hydrophilic interaction (normal phase partition)
Bare silica gels
with methanol- or acetonitrile-rich eluent*
with eluent impregnated with polyamine as *pseudo* NH_2-bonded phase*
Aminopropylated silica gels
with acetonitrile-rich eluent *
Amide-, polyamine-, diol- or polyol-bonded silica gels
as an alternative to aminopropylated silica gels
Cation-exchange resins (hydrogen form)
with aqueous ethanol
Hydrophobic interaction (reverse phase partition)
Octadecyl, decyl-bonded silica gels
with plain water as eluent*
Charge-transfer
Graphite
with water or dilute aqueous ethanol
Ion-exchange
Anion-exchange resins
with strong alkali (as anions, coupled with pulse amperometric detection)
with borate buffers (as borate complexes)
Cation-exchange resins
with dilute acids (for amino sugars)
Ligand exchange
Cation-exchange resins (metal forms)
with water
Size exclusion
Cross-linked polysaccharides
with water or dilute buffers

*applied also for CEC of carbohydrates

raphy nowadays occupies the mainstream for the separation of carbohydrates, though various means are required to conquer the problem of their high hydrophilicity. Since it is important to overview HPLC of carbohydrates in order to understand the current states of and the future of CEC of carbohydrate, Table 1 summarizes the separation modes and systems reported for HPLC of intact carbohydrates.

2.1. Separation

At the early stage of HPLC of carbohydrates, normal phase partition mode (hydrophilic interaction) was preferentially used for the separation of mixtures of mono- and simple oligosaccharides. Bare silica packings were used for this purpose, but silica gels have a fundamental problem that they are considerably soluble in aqueous solvent, especially in acidic or basic media and at elevated temperatures [5]. Therefore, methanol- or acetonitrile-rich (>80%) aqueous eluent was preferably used for the separation of monosaccharides [6]. The durability of bare silica packings could be enhanced by using non-aqueous media such as a dichloromethane-methanol mixture [7]. Silica gel columns were also used for the separation of hydrophobic glycoconjugates such as glycolipids [8].

Chemically bonded silica phase, such as aminopropyl silica could improve the limitation of bare silica. A detailed study by Niklov and Reilly [9] indicated that the selectivity of aminopropyl silica is different from that of bare silica, and it also could separate oligosaccharides of various sizes. For example, maltooligosaccharides were successively resolved up to 30mers by isocratic elution [10]. There have been many applications for the separation of glycoprotein-derived glycans [11-13]. Though aldoses have a tendency to form glycosylamines in columns of aminopropyl silica, such phenomenon could be avoided by adding a neutral buffer to an eluent [14], or reduction of aldose samples to alditols. Amide- and polyamine-bonded silica gels were similarly used for hydrophilic interaction chromatography of intact carbohydrates [15]. *In-site* hydrodynamic coating of bare silica columns by adding a small quantity (<0.1%) of polyamines, such as 1, 4-diaminobutane to the eluent [16] is an alternative to provide polyamine columns. Good separation of tritium-labeled reduced oligosaccharides from high-mannose type glycans of a glycoprotein was reported by Turco [17]. Normal phase columns of diol [18] or polyol-bonded silica [19] were also available, but the separation sometimes required elevation of column temperature [20].

Separation of highly hydrophilic carbohydrates was possible on ODS columns of low coverage using plain water as eluent [21]. Although this system seems to have limitation for intact carbohydrates, good resolution was obtained for permethylated and peracetylated carbohydrates [22, 23]. Cation-exchange columns of the hydrogen form can also be used as substitute for ODS columns, but addition of aqueous ethanol is often necessary [24].

Use of graphite carbon columns (GCC) is one of the recent topics of carbohydrate separation. There was a report that cyclomaltooligosaccharides with varying dps were better separated on a GCC column than on an ODS column [25]. Separation on GCC differs with its batches and the separation mechanism has been considered to be due to charge-transfer. Good separation was also obtained for glycoprotein-derived neutral high-mannose type glycans [26] as well as sialylated acidic glycans [27]. However, whether the carbon column can generate the electroosmotic flow (EOF) in the electric field is not known.

Anion-exchange on latex-type anion exchange columns in strong alkaline solutions has been used as a separation mode for carbohydrates, combined with pulse amperometric detection [28]. The hydroxyl groups in carbohydrates are partly dissociated to give alkoxide ions in such alkaline media, and carbohydrates can be separated by the difference in charge and size. There have been many reports on this system applied for the separation of

glycoprotein-derived glycans [e.g., 29]. In this system elution of larger oligosaccharides required stronger alkaline solutions.

Neutral carbohydrates form anionic complexes with borate ions, which can be separated on anion-exchange columns with borate buffer as eluent. Ligand-exchange chromatography is based on the complex formation between the hydroxyl groups of carbohydrates and multivalent metal ions such as Pb^{2+} or Ca^{2+} trapped on a cation exchange resin. Separation of monosaccharides can be attained with plain water as eluent. In open column liquid chromatography oligo- and polysaccharides can be separated by size-exclusion using cross-linked polysaccharides, but these materials are so soft that they cannot endure high pressure in HPLC. Cation-exchange resins can separate smalloligosaccharides by this mode, but the separation needs heating the column at 60 - 80℃.

2.2. Detection

Carbohydrates generally have neither chromophore nor fluorophore. Absorption at low wavelengths (<200 nm) or indirect detection by fluorescence quenching may be useful in some cases. Refractive index detector is often used for the detection of carbohydrates. More recently light-scattering detection has been recommended by several authors [30]. Though these detecting methods are simple, they have the major disadvantages that they are not selective to carbohydrates. Such a drawback may be avoided by converting carbohydrates to fluorescent or UV-absorbing derivatives. A number of reagents have been reported for pre-column derivatization as shown in Figure 1. These reagents can be classified into four categories based on the type of derivatization reaction (Figure 2). The condensation of the reducing end of a reducing carbohydrate with the amino or the substituted amino group in the presence of a suitable reductant gives a glycamine (Category 1) probably *via* a glycosylamine. The labeling method using AP was first reported by Hase *et al.* [31] and now widely applied for mapping *N*-linked glycans derived from glycoproteins [32]. ABEE [33], AMAC [34], ANTS [35], and APTS [36] are also frequently used for the same purpose. The formation of hydrazones (Category 2) is also used for derivatization of reducing carbohydrates, though this category has a problem of *syn/anti* isomerism. The glycamines, formed by reductive amination of reducing carbohydrates with a salt of ammonia or methylamine, can be condensed with various reagents to give characteristic derivatives (Category 3). The derivatives formed by condensation with NBD-F [37] and CBQCA [38] are notable, because they can be sensitively detected by LIF. PMP [39] and its *p*-methoxyphenyl analog (PMPMP [40]) are attractive reagents for derivatizing reducing carbohydrates, especially sialylated oligosaccharides, since the derivatization proceeds under mild conditions and does not cause loss of the sialic acid residues (Category 4). Although a number of tagging reagents have been developed for the

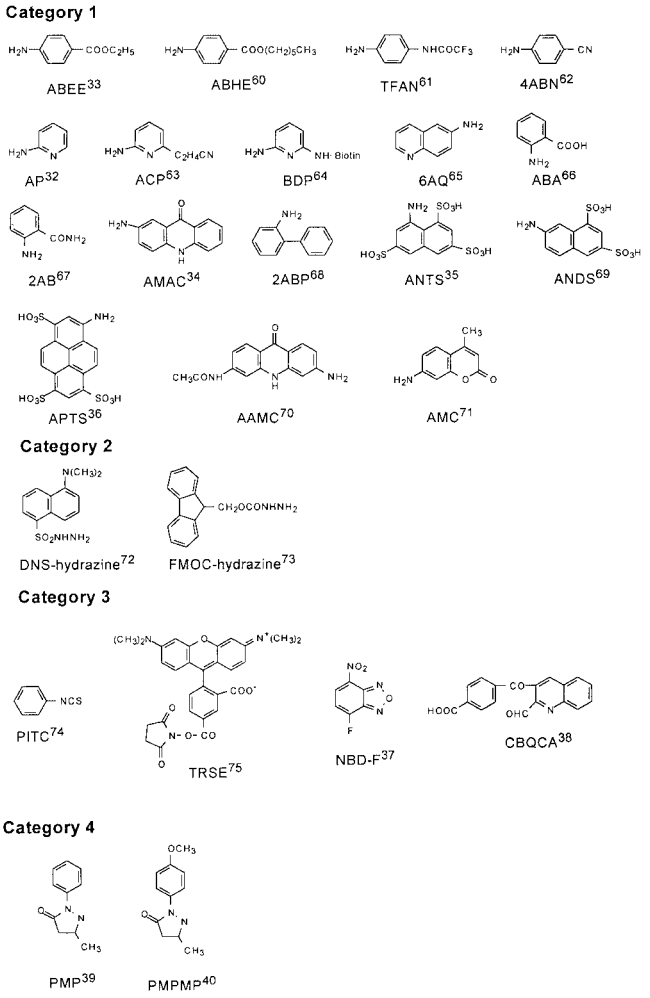

Figure 1. Various reagents for pre-column labeling of carbohydrates. ABEE: ethyl p-aminobenzoate, ABHE: hexyl p-aminobenzoate, TFAN: trifuluoroacetamidoaniline, 4 ABN: 4-aminobenzonitrile, AP: 2-aminopyridine, ACP: 2-amino-6-cyanoethylpyridine, BDP: biotinylated diaminopyridine, 6AQ: 6-aminoquinoline, ABA: 2-aminobenzoic acid, 2AB: 2-aminobenzamide, AMAC: 2 -aminoacridone, 2ABP: 2-aminobiphenyl, ANTS: 8-aminonaphthalene-1,3,6-trisulfonate, ANDS: 7-aminonaphthalene-1,3-disulfonate, APTS: 8-aminopyrene-1,3,6-trisulfonate, AAMC: 3-acetylamino-6-aminoacridone, AMC: 7-amino-4-methylcoumarin, DNS: dansyl-, FMOC-: fluorenylmethoxycarbonyl-, PITC: phenylisothiocyanate, TRSE: 4-carboxytetramethylrhodamine succinimidyl ester, NBD-F: 4-fluoro-7-nitrobenz-2-oxa-1, 3-diazole, CBQCA: 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde, PMP: 1 -phenyl-3-methyl-5-pyrazolone, PMPMP: p-methoxy-PMP.

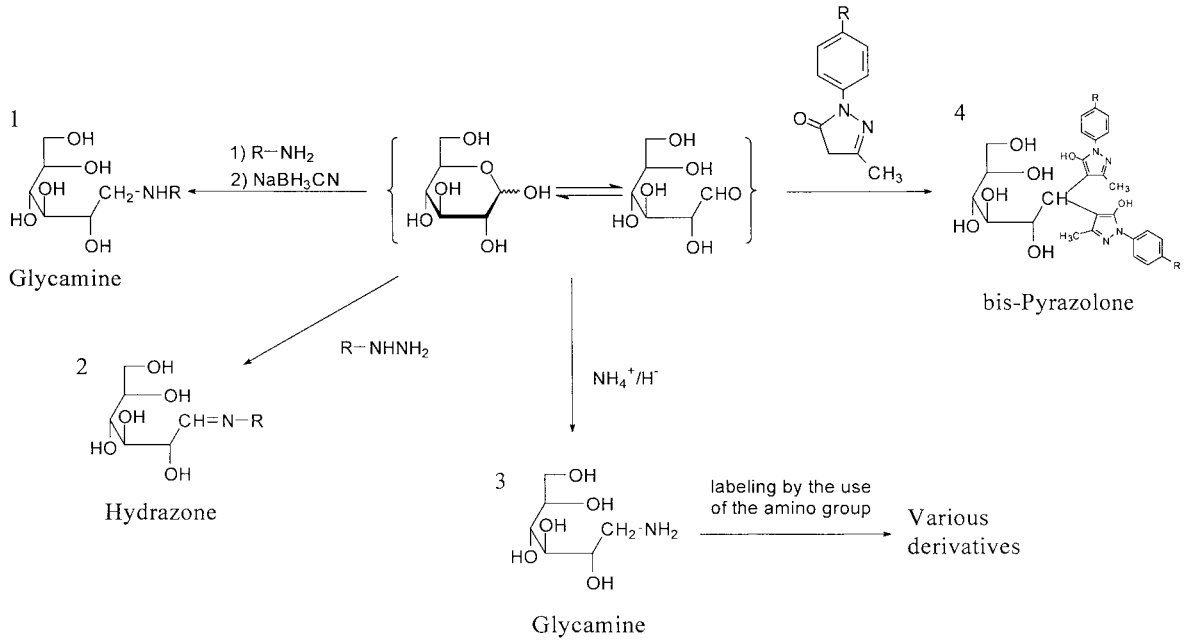

Figure 2. Various reaction types for pre-column derivatization of carbohydrates. The arabic numbers on the upper left of each structure indicate category numbers.

sensitization of carbohydrates, only PMP has been used in the CEC analysis of carbohydrates. Introduction of tags to carbohydrates causes change of physico-chemical properties of the original carbohydrates. In most derivatives hydrophobicity increases. This is desirable for separation by hydrophobic interaction on ODS, but the derivatives formed with the reagents in Categories 1-3 are positively charged in neutral to weakly acidic media, which giving rise to an undesirable effect on separation as mentioned in 3.2.

Apart from sensitization by derivatization, pulse amperometric detection on a gold electrode is an attractive method for carbohydrate analysis. It has been widely used in combination with an anion-exchange separation with strong alkaline eluent. There have been many reports on HPLC [29] as well as capillary electrophoresis [41, 42] using this system, but no papers have appeared yet on CEC of carbohydrates. This is because the use of such strong alkaline eluent will require special columns durable to repeated analysis.

3. Current status of CEC

3.1. Underivatized carbohydrates

HPLC on reverse-phase columns with direct detection is generally not a good strategy for carbohydrate analysis, because separation is limited and detection sensitivity in UV absorption is not high. Zhao and Johnson [43] challenged CEC analysis of sucralose (4, 1', 6'-trichlorogalactosucrose), a non-nutritional sweetener, and related compounds in its manufacturing process. The column and eluent used were Hypersil ODS (particle size, 3 μm ; column dimension, 100 μm i.d., 250 mm) and an acetonitrile-4 mM borate buffer (pH 9.2) mixture (25 : 75, v/v), respectively. Separation was good owing to a rather hydrophobic nature of the solute by the presence of the halogen atoms. Formation of borate complexes should be taken into account, but it is considered to give only a minor effect under such a low borate concentration. Detection at 195 nm gave satisfactory sensitivity for this process substance analysis.

There was another example for CEC of intact carbohydrates by Guo and coworkers [44]. In this case mono- and disaccharides separated on an ODS column was detected by a condensation nucleation light scattering detector (CNLSD). In this detection the outlet end of the separation column was modified to effect vaporization. Removal of the polyimide coating, followed by spraying the revealed silica surface with gold paint facilitated electrospray at the outlet. Supplementary pressure was applied to the eluate to stabilize the electrospray process. Good reproducibility was obtained and the detection limit reached 6 to 9 pg for galactose, glucose, fructose, and sucrose. Change of applied pressure/voltage ratio caused alteration of column efficiency and also separation mode.

The sensitivity in the forgoing two examples of CEC of intact carbohydrates was satisfactory but both methods are not selective to carbohydrates, hence the analysis may be interfered by other compounds in sample.

3.2. Derivatized carbohydrates

Derivatization of carbohydrates with various reagents enhance detection sensitivity, but at the same time gives influence on the re-

tention and resolution of carbohydrates, especially on ODS columns. Among various kinds of derivatives substituted glycamines prepared by reductive amination seem to be strongly adsorbed on the remaining silanol groups, resulting in peak broadening, and accordingly poor separation. Such ionic interaction may be suppressed by using eluent containing higher concentrations of electrolytes (our unpublished results).

In contrast to these glycamine derivatives, PMP derivatives have rather weakly acidic nature. Therefore, separation can be achieved on chemically bonded silica with low coverage with low electrolyte eluents. The authors group reported the CEC of PMP derivatives [45, 46], in which all members of the D-series of aldopentose epimers (Ara, Lys, Rib, and Xyl), and the component monosaccharides in glycoproteins (Fuc, Gal, Man, GlcNAc, and GalNAc) were well separated from each other on a Hypersil ODS column (particle size, 3 μm ; column dimension, 100 μm i.d., 255/300 mm) with a 50 mM HEPES (pH 6.0 to 6.3) -acetonitrile mixture (2.2 : 1, v/v) as eluent. The relative standard deviation (RSD) of elution time was 0.6% (n = 5) and the RSD value for relative peak response to 3-*O*-methyl-D-glucose was less than 5%. More recently Gucek and Pihlar [47, 48] similarly succeeded in the resolution of Man, Lac Rib, Glc, and Gal as their PMP derivatives by using a Hypersil ODS 1 column (particle size, 5 μm ; column dimension, 100 μm i.d., 30/50 cm) and 5 mM monosodium phosphate dissolved in acetonitrile water (3 : 1, v/v).

Since electroosmotic flow (EOF) is a driving force in CEC, the rapidity of analysis in CEC depends on the velocity of EOF, which is generated mainly due to the electric double layer between the particles of supporting materials and eluent. The zeta potential on the surface of silica gel particles or modified silica gel particles becomes higher as the population of the silanol group becomes more abundant. Therefore, the use of ODS completely modified by endcapping as used for HPLC is not advantageous for CEC. In our experience almost no peaks appeared within 1 h, when we analyzed the PMP derivatives of some monosaccharides on an endcapped ODS column (unpublished results). The use of modified silica having reasonable proportions of the unprotected silanol group is better for CEC. Yang and El Rassi [49] prepared an ODS stationary phase with low surface coverage by controlling reaction of bare silica with an octadecyl donor. The introduced amount of the octadecyl group was estimated to be only 2.1 μmoles/m^2, which means that 25% of the silanol group remained unmodified. EOF was dependent on the amount of the packed gels. Thus, the velocity of EOF generated from a fully packed column was 0.62 mm/s, whereas that from a half packed column was 0.38 mm/s. Several *p*-nitrophenyl glycosides of monosaccharides and maltooligosaccharides could be separated from each other with an acetonitrile -2.5 mM phosphate buffer (pH 6.0) mixture (2 : 8, v/v). The α- and β-anomers of *p*-nitrophenyl glycosides were readily resolved with an eluent containing borate buffer.

Reports on CEC of derivatized carbohydrates are quite few at this point as mentioned above, and they are restricted to PMP derivatives and *p*-nitrophenyglycosides, which have no amino/imino functions. Column efficiency of ODS columns are generally not high, the height equivalent to a theoretical plate (H) being in a range of 53-60 μm. In addition peaks sometimes show a tailing tendency. The low column efficiency is considered to be due to the hydrophilic interaction with the silanol group, as generally observed on ODS columns in HPLC. Thus, the needs for unprotected silanol groups for sufficient velocity of EOF gives negative effect for column efficiency and accordingly separation of derivatives of carbohydrates.

Organic polymer-based stationary phases will overcome this problem of unfavorable hydrophilic interaction on silica-based reverse-phase packings. Palm and Novotny [50] introduced an *in-situ* prepared macroporous organic polymer column (continuous beds) for CEC of carbohydrates. The inner wall of a capillary column was treated with a bifunctional silane reagent (Bind silane^R) prior to polymer preparation. A mixture of acrylamide and bisacrylamide (4% T, 60% C) (for preparing strong support), either acrylic or vinylsulfonic acid (for generation of suitable EOF), either butyl, hexyls or lauryl acrylate (for obtaining reasonable hydrophobicity), and polyethylene glycol (for preventing turbidity) were co-polymerized inside the capillary. A sample of maltooligosaccharides was reductively aminated with 2-aminobenzamide in this case, to prepare derivatives detectable by He-Cd laser (325 nm)-induced fluorescence. As shown in Figure 3, good separation was observed by using a capillary column prepared with butylacrylate and vinylsufonic acid, and by using an acidic eluent.

Size separation of glucose to maltoheptaose was achieved within 12 min with H values of 4.4-5.3 μm. Although this method requires a tailor-made column and a special device for LIF detection, it was proved useful for high-resolution separation.

Another strategy to conquer the problem of peak broadening and tailing is the use of hydrophilic interaction of derivatized carbohydrates as the main separation mode. As already pointed out for HPLC of carbohydrates, this mode can be achieved by using either a bare silica column or an aminopropylated silica column with a bufferized aqueous acetonitrile as eluent. Figure 4 shows the separation of PMP derivatives of five monosaccharides commonly found in glycoproteins (Fuc, Gal, Man, GlcNAc and GalNAc) on a bare-silica (Develosil silica, 30 Å, 3 μm)-packed capillary (100 μm i.d., 24.5 cm) [51].

Due to fast EOF, separation was achieved in only 8 min.

It should be noticed that some modified silica gels having ionic groups, such as aminopropyl silica gels, generate a bulk flow

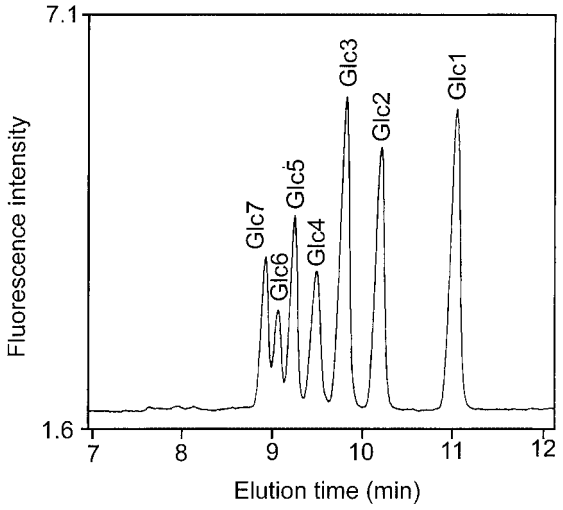

Figure 3. Isocratic CEC of maltooligosaccharides (dps 1-6) as their 2-aminobenzamide derivatives in a capillary filled with a macroporous polyacrylamie/poly (ethylene glycol) matrix, derivatized with C_4 ligand (15%) and containing vinylsulfonic acid (10%). Capillary size, 100 μm i.d., 25 cm; eluent, acetonitrile-10 mM Tris/15 mM boric acid (pH 8.2) mixture (2 : 8, v/v); field strength, 900 V/cm; sample concentration, 5-10 μM. Reproduced from Ref. 50 with the permission of the publisher.

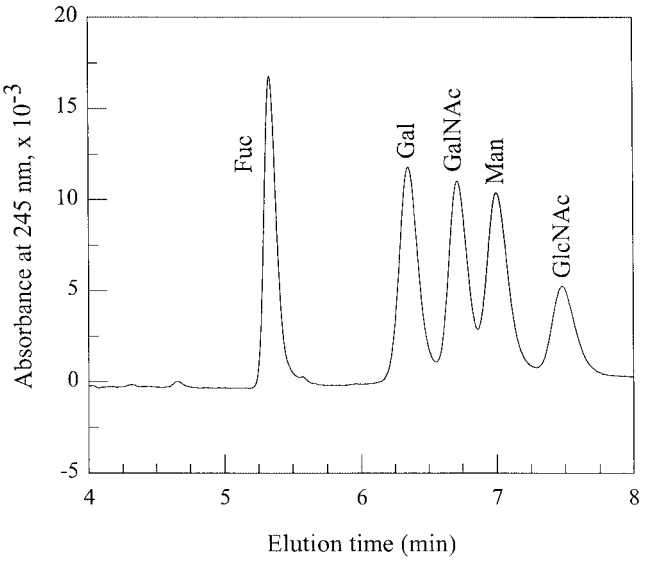

Figure 4. Analysis of the monosaccharides present in glycoproteins as PMP derivatives. Column, Develosil 30 (particle size, 3 μm; column dimension, 100 μm i.d., 24.5 cm); eluent, acetonitrile -50 mM HEPES buffer (pH 6.0) mixture (4 : 1, v/v); applied pressure, 7.5 kg/cm^2; applied voltage, 20 kV; column temperature, 20℃; detection, 245 nm; sample, a mixture of PMP-sugars (10 mM each); sample injection, 5 kV, 5 s.

from cathode to anode, when packed in an uncoated capillary. On the other hand, the silanol groups on the capillary inner-wall generate a flow toward the opposite direction (anode to cathode). Due to this counter flow analysis time of the solutes increases and column efficiency is reduced. We attempted preparation of tailored columns of chemically bonded silica by pumping an ethanolic solution of a silylating reagent, such as aminopropyltrimethoxysilane into a capillary packed with 3 μm-bare silica particles [52]. The silylation reaction was complete in 1 h by heating the column. Such in-capillary reaction resulted in modification of not only packed silica gels but also the capillary inner wall. The reproducibility of preparation was high; aldopentoses as PMP derivatives were completely separated in 10 min with an acetonitrile-100 mM HEPES (pH 6.5) mixture (3 : 1, v/v) as eluent. The resolution was superior to the column prepared by packing a commercial preparation of 3 μm-amino silica gels into an uncoated capillary (analysis time, 20 min). We also attempted preparation of another type of amino silica column, similarly by flowing a dimethyloctadecyltrimethoxysilylammonium chloride solution through a bare-silica packed column. The resultant column generated faster EOF to the anode, and the resolution of the PMP sugars was slightly better than on the aminopropylated silica column mentioned above.

3.3. Carbohydrate-related compounds

Carbohydrates exist not only in free state but also in conjugation with other compounds, such as proteins and sphingonolipids, to form glycoproteins and glycosphingolipids, respectively. Conjugation with small molecules such as phenolic compounds and triterpenes gives various glycosides. From biomedical importance of these glycoconjugates, analysis by CEC has been investigated. A high resolution separation of cardiac glycosides, belonging to triterpene glycosides, was reported by Meyer and coworkers [53]. In this case a special device was used to obtain high column efficiency. Frit fabrication is one of the important technical problems in the preparation of columns for CEC. Frits prepared by hitherto developed techniques suffer from physical weakness to handle, low reproducibility in preparation, and nucleation stimulating bubble formation. Meyer and coworkers packed 1.5-μm nonporous ODS silica gels (NPS ODS II) into a capillary (100 μm i.d., 24.5 cm) tapered by a laser-based micropipette puller. The tapered end could be used as the inlet of CEC column after packing stationary phase. The outlet of the stationary phase did not require fabrication of a frit, because the particles were retained in the column by electrostatic attraction towards the anode under the operating conditions of CEC. This non-frit technique enabled high-resolution efficiency of more than 500,000 plates/m. Separation of three cardiac gly-

cosides was attained within 4 min.

It is well known that many drugs and toxic compounds in animal bodies are metabolized to their glucuronides. Hugener and coworkers [54] adopted the combination of CEC and MS for the analysis of some aromatic glucuronides. A mixture of phenyl, *o*-aminophenyl, *p*-nitrophenyl, and α-naphthyl glucuronides was separated into its components by pressurized CEC on a Hamilton RPR-1 (12-20 μm) packed column (220 μm i.d., 20 cm) with an acetonitrile-2 mM ammonium acetate (pH 7.0) mixture (5 : 95, v/v) as eluent. Detection was performed by mass spectrometry *via* thermospray interface by the negative ion mode. Stepwise application of voltages realized reduction of analysis time to *ca*. 30 min.

Glycosphingolipids are amphiphilic compounds composed of a polar moiety of carbohydrates and hydrophobic parts of fatty acids and sphingosine. The structural diversity of glycosphingolipids is mainly due to the carbohydrate chains varying in size from monosaccharide to large branched oligosaccharides. The glycosphingonolipids are known to form stable mixed micelles (C.M.C. = 10^{-10}~ 10^{-8} M) under biological conditions. Therefore, in capillary electrophoretic analysis, either organic solvent or a complexation reagent such as cyclodextrins [55] was added to prevent the micelle formation. Zhang and El Rassi prepared an octadecylsulfonated silica (ODSS) by introducing a hydrophilic sulfonate compound and a hydrophobic octadecyl compound to the γ-glycidoxypropyltrimethoxylsilane-treated silica gels [56], and analyzed sialic acid-containing glyconosphingolipids, (gangliosides) on the resultant ODSS [57]. Resolution of gangliosides G_{M1a}, G_{D1a}, G_{D1b}, and G_{T1b} was good, when a column packed with 5-μm ODSS having a porosity of 120 Å was used, eluted with a methanol-acetonitrile-10 mM borate (pH 9.40) mixture (50 : 30 : 20, v/v). Methanol was essential for the separation of G_{D1a} and G_{D1b}, and complete resolution was achieved by using borate concentrations of more than 8 mM. A mixture of neutral glycosphingonolipids including galactosyl-, lactosyl-, globotriosyl- and globotetraosylceramide were strongly retained on this column, but fine resolution was achieved by using a non-porous ODSS column and a tetrahydrofuran -2 mM ammonium phosphate (pH 7.0) mixture (80 : 20, v/v) as eluent.

Cancer patients exhibiting advanced muscle proteolysis secrete a specific sulfated glycoprotein called the cachectic factor in urine. This glycoprotein consists of a short peptide chain (~4,000 *Da*) extensively glycosylated at the Asn and Ser residues. The carbohydrate chains are sulfated and these sulfated *N*- and *O*-linked glycans have been found to be antigenic determinants [58]. Choudhart and coworkers [59] compared three microanalytical separation methods including capillary electrophoresis, capillary liquid chromatography, and CEC (with or without pressure) using two types of columns packed with Zorbax ODS (3.5 μm) and Hypersil ODS (3 μm). Since this cachetic factor was highly hydrophilic, retention on the Hypersil ODS column, the more hydrophilic stationary phase, was more marked. Thus, the glycoprotein peak appeared at about 17 min on the Zorbax ODS column and at 32 min on the Hypersil stationary phase. There was no great difference among these three separation methods, but the cachectic factor could be separated from other components by CEC.

4. Conclusion

Although CEC has been accepted as a powerful tool for separation of biomedical substances, only a small number of papers have been published for carbohydrate analysis. Since CEC is a kind of chromatography performed in the presence of EOF, the accumulated results of HPLC will be of great help for better separation/detection systems for CEC.

The major materials for stationary phase are silica gels and modifications. In order to generate EOF rapid enough to drive separated solutes, the surfaces of the stationary phase as well as capillary inner wall must be electrically charged to a considerable extent. This means that a considerable part of the silanol group should be left unprotected. It is an irony that this reminder plays a negative role for column efficiency. Therefore, although CEC utilizes EOF as driving force, which is advantageous for high column efficiency by its plug flow nature, the hydrophilic and other interactions between the solutes and the silanol group spoil this high capability of EOF.

From these reasons development of materials other than silica gels and modifications will be of keen interest. As already demonstrated by Palm and Novotny [50], use of organic polymers will be one of the promising tactics. Continuous bed will be the ultimate style of stationary phase. On the other hand detection in CEC will follow up the technique in capillary electrophoresis. Thus, UV detection will be most suited for routine analysis, and LIF detection can minimize sample scale. Sample reduction will be beneficial for better resolution of solutes.

Since CEC requires packing of stationary phase in narrow capillaries, the technique to stabilize the beds in the capillaries is important. Preparation of a frit at the outlet of capillary prior to packing has been the standard procedure for CEC, but the preparation of frits by this technique is not easy, and inadequately prepared frits cause low reproducibility of solute elution. The use of a tapered capillary by Guo and coworkers [43] is notable, because it did not need any frit. Such fritless packing will be one of the solutions for this problem.

Due to the scarcity of published papers other reviews on CEC of carbohydrates seem to have not yet appeared. We are interested in the development of this area in the near future.

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