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Review

Carbohydrate analyses with high-performance anion-exchange chromatography

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Abstract

High-performance anion-exchange chromatography (HPAEC), in tandem with pulsed amperometric detector (PAD) is a powerful tool for carbohydrate analysis. It requires no pre- or postcolumn derivatization, and yet offers superb resolution and sensitivity. In its basic mode, alcoxide anions from the hydroxyl groups of carbohydrates as well as other anionic substituens are utilized. In addition to the number of hydroxyl groups, separation can be based on anomers, positional isomers, as well as degree of polymerization.

Contents

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1. Introduction

The advent of high-performance anion-exchange chromatography system (HPAEC) in the late 1980s revolutionized carbohydrate analyses. The major advantages of HPAEC are: (i) speed of analysis (for example, monosaccharide analysis can be done within 15 min); and (ii) high resolution (e.g., separation of anomeric and positional isomers). These were achieved by the superior design of "MicroBead pellicular resin" in which small anion-exchange resin spheres are ionically attached to a larger cation-exchange resin sphere. Moreover, sub-nmol range sensitivity without derivatization is feasible with the technology of "pulsed amperometric detector" which is an important adjunct to the HPAEC. Frequently a compound term "HPAEC–PAD" is used to describe the combination of the chromatographic system and the detector.

Prior to the invention of HPAEC, anion-exchange chromatography had been used to separate anionic carbohydrates as such and neutral carbohydrates in the form of borate complexes. What is defined as "High-performance Anion-exchange Chromatography" may also be interpreted as "High pH Anion-exchange Chromatography" because of the almost inevitable use of highly alkaline eluents in this form of chromatography. The latter interpretation would exclude, for example, separation of polyanionic carbohydrates such as polysialic acid by DEAE columns at neutral or acidic pH. However, sometimes the use of neutral or acidic pH can also be beneficial even when pellicular resin is used, and thus the boundary of the definition would become blurred.

A few reviews have been written on the subject of HPAEC of carbohydrates [1,2]. In this article, generalizations stated in the previous reviews are summarized only briefly, and more recent developments will be examined in greater detail.

2. Acidity of analytes

Other things being equal, retention times are closely related to the pK_a of the analytes. Obviously, an acidic carbohydrate such as mannose-6-phosphate is retained much longer on the column than mannose itself, and neutral, monosialyl, disialyl, and trisialyl oligosaccharides are eluted in that order. These phenomena are also observed in the earlier anion-exchange chromatography, where the main acidic groups used in the chromatographic process are those of stronger acid (e.g., phospho-, sulfo-, or sialyl) derivatives), or which are anionic under neutral or weakly acidic pHs.

HPAEC can do much finer discrimination than that obtainable by using the common acidic groups only, because it utilizes the subtle differences in the pK_as of the OH-groups of carbohydrates. The dramatic separation of N-glycolyl-neuraminic acid from N-acetyl-neuraminic acid is an example [1]. Separation of galactose, mannose, and glucose are possible by HPAEC despite similarities in their overall molecular size and the number of hydroxyl groups [1].

The reduced retention of alditols as compared to the parent reducing sugars can be readily understood on the basis of acidity. Since the anomeric hydroxyl group is the most acidic among all the OH-groups, replacing it with an ordinary OH-group (which is less acidic) would naturally result in less retention. Inositols are not well retained because of their lack of acidic anomeric hydroxyl group. Alkyl glycosides are not retained as much as the parent sugars for the same reason.

Likewise, the loss of hydroxyl groups, such as in rhamnose or fucose, reduces retention times. This also applies to the amino sugars, in which an OH group is replaced with amino or acetamido groups. (The N-acetyl-chito-oligosaccharides present a special case, which will be discussed later.)

3. Molecular size and shape

For homologous sugar series, as the number of carbons (or hydroxyl groups attached to them) increases, there are increases in retention time [3]. For example, tetraoses are retained longer than trioses.

In a homologous oligosaccharide series, it can also be generalized that the longer the degree of polymerization (DP), the longer the retention time. When $\log(k')$ —where k' is elution volume/void volume— is plotted against DP, a linear relation is usually obtained [4]. Positional isomers, such as isomaltose (Glcα6Glc) and maltose (Glcα4Glc), and anomeric isomers, such as maltose (Glcα4Glc) and cellobiose (Glcβ4Glc), are also well separated. When a series of gluco-oligosaccharide homologs of different linkages are compared, it is possible to deduce their inter-Glc linkages [4,5], because the "ladder patterns" are different between polysaccharides of different linkages. Among the glucose-oligosaccharides, α-1,3 and β-1,3 series showed the greatest unit increment, and α-1,6 series (isomaltose) showed the smallest unit increment [4].

The component sugars also play a big role. For example, the unit increments observed for the -(Xylβ4)$_n$- series and the -(Manβ4)$_n$- series were much smaller than that for the -(Glcβ4)$_n$- series, despite their apparent similarities in linkage mode and overall conformation. No satisfactory explanation can be given for these observations.

One of the unique structural characteristics of carbohydrates is their ability to form branched structures. Many lectins can sensitively discern branch isomers. For example, mammalian hepatic lectins [6] can discriminate the two isomeric triantennary structures of bovine fetuin. These isomeric triantennary oligosaccharides can also be separated by HPAEC [7]. Isomers formed by sialylation in different positions, such as α-2,3- and α-2,6-sialyl lactose, are easily separable. Another excellent example of the high resolution is shown in the separation of positional isomers of branched cyclodextrins [8]. The same type of separation was accomplished for positional isomers of phosphate esters [9,10] and sulfate esters [11]. Among the mono-phosphates of D-glucose, the 1-phosphate elutes the earliest, as expected [10]. The separation of sugar nucleotides, nucleotides, and sugar phosphates were also accomplished with an OmniPac PAX-100 column [12].

4. Chito-oligosaccharides [-(Glcβ4)$_n$- series]

4.1. Abnormal retention times

On a CarboPac PA-1 column, di-N-acetyl-chitobiose is retained somewhat longer than GlcNAc, but retention times actually decrease for oligosaccharides larger than triose. Somehow certain structural features in the segment of GlcNAcβ4GlcNAc are not favorable for retention on the PA-1 column. This is also reflected in the fact that when the N-glycosides are enzymatically released by cleavage of the glycosidic linkage in the GlcNAcβ4GlcNAc segment of the core (with endo-H or endo-F), the resultant oligosaccharides are retained longer than when the same oligosaccharides are released by glycoamidase (e.g., glycopeptidase F) action [13], retaining the GlcNAcβ4GlcNAc segment. Since the Manβ4GlcNAcβ4GlcNAc structure in the core resembles tri-N-acetyl-chitotriose and the oligosaccharides of the Manβ4-series also show no perceptible increase as degree of polymerization (DP) increases [1], the change from Manβ4GlcNAcβ4GlcNAc to Manβ4GlcNAc perhaps is analogous to the change from chitotriose to chitobiose, which actually causes the increase in the retention time despite decrease in the number of sugar residues and the OH-groups.

4.2. The effect of 6-α-fucose linked to GlcNAc

Oligosaccharides containing Fucα6GlcNAc at the reducing teminus are eluted earlier than those without the Fuc [14,15]. The effect of Fuc linked to GlcNAc at or near the terminal of branches has been also noted [7,16]. These effects of fucose on the retention time are reminescent of those found for reversed-phase HPLC of 2-aminopyridine derivatized oligosaccharides (PA-oligosaccharides) [17].

5. Quantification and selectivity

Quantification of the analyte by pulsed amperometric detector (PAD) is very convenient, because it does not require any pre- or post-

column derivatization. However, unlike radio- or fluorescence-labelled oligosaccharides, the molar response of analyte measured with PAD is somewhat different for each compound. In a simple case such as analysis of monosaccharides, where pure reference compounds can be obtained readily, this problem can be overcome by establishing a calibration line for each of the compounds being analyzed. However, in most cases of oligosaccharide analysis, the reference compounds are either unknown or unavailable in pure form or not available in sufficient quantities. Accurate quantification of such carbohydrates can be quite difficult or impossible, and investigators often have to settle for a "relative quantification."

6. Epimerization and degradation

The problem of undesirable side-reactions caused by strongly alkaline elution conditions have been discussed in a previous review [1]. In 100 m*M* NaOH, the condition frequently used in HPAEC elution, the equilibrium of epimerization of GlcNAc to ManNAc is virtually reached within an hour. It is thus not too surprising that some large oligosaccharides containing GlcNAc or GalNAc as reducing terminus would suffer from epimerization and degradation. This is partly the reason why some workers use reduced oligosacharides (see below). If the reducing terminus of the oligosaccharide contains alkali-sensitive sequence, such as Galβ3GalNAc, the side reaction is usually not limited to epimerization, and the evidence of degradation (e.g. β-elimination) becomes apparent during the elution. There is no real solution to the problems of alkali-mediated side reactions but to reduce the elution time or use a lower concentration of hydroxide ion, and compensate with higher acetate or nitrate.

7. Reducing vs. reduced mono- and oligosaccharides

Whether to use reducing oligosaccharides or reduced oligosaccharides (alditols) for HPAEC analyses has been debated since the introduction of HPAEC. The main reason for reduction of oligosaccharides prior to HPAEC analyses is to prevent unwanted epimerization or degradation when a very strongly alkaline condition must be used for elution. Although the epimerization and degradation during the HPAEC runs may not present perceptible or serious problems, the use of reduced oligosaccharides results in better defined peak shapes [14,18]. On the other hand, reduction of oligosaccharides requires additional steps, and complete reduction and recovery of all the reduced oligosaccharides in the sample must be ascertained. The previous studies indicated that the alkaline conditions generally used for reduction (e.g., 0.5 *M* sodium borohydride in 0.1 *M* NaOH) may cause epimerization and degradation of some alkali-sensitive oligosaccharides (e.g., those containing Galβ3GalNAc at the reducing terminus). Another disadvantage of using reduced oligosaccharides is that it is very difficult to attach other probes if the separated oligosaccharides are to be examined by other methods such as reversed-phase HPLC or capillary zone electrophoresis (CZE) which requires that a chromophore or flurophore be attached to the oligosaccharides. If the reduction step is carried out with tritium-labeled $NaBH_4$, all subsequent quantification would not be dependent on PAD detection, thus avoiding the problem of dealing with differential PAD-responses for various oligosaccharides. This is basically the approach used by Kobata and his co-workers [19], although chromatographic systems other than HPAEC were used.

Sometimes, the nature of samples is such that only reduced oligosaccharides can be used. The O-glycosides in glycoproteins are most frequently released by alkaline β-elimination in the presence of a reducing agent such as $NaBH_4$, and the released oligosaccharides are then in reduced form. In the analysis of oligosaccharides derived from some glycosaminoglycans [20], reduction is needed for stability. For these problems, the new CarboPac MA-1 column specially designed for alditols may be helpful (see Section 9: Newer columns and instrumentation).

8. O-linked oligosaccharides (O-glycosides)

The O-glycosides in glycoproteins are frequently released by alkaline β-elimination under reductive conditions. Unfortunately, analysis of alditols presents some problems to HPAEC. The loss of the acidic anomeric OH-group definitely cause a decrease in retention time. This is especially troublesome when the size of the reduced oligosaccharides are small (di- or trisaccharides).

8.1. Reduced and desialylated O-glycosides

Neutral oligosaccharide alditols derived from mucin glycoproteins have been effectively separated [21]. However, the compositional analysis of the oligosaccharides with terminal N-acetyl-galactosaminitol is quite difficult because N-acetyl-galactosaminitol is hardly retained on the PA-1 column. This problem is solved by succinylation of the amino group in the hexosaminitol liberated after complete hydrolysis, so that N-succcinylated hexosaminitol is retained much longer [22]. The succinylation also improves the retention and resolution of short reduced oligosaccharides containing aminosugars or their alditols.

8.2. Reduced sialylated O-glycosides

If the alditols obtained by β-elimination are sialylated, then chromatographic analysis becomes much easier because of the greater retention times [22]. There have been some reports of analyses of sialylated O-glycosides released by reductive β-elimination [22,23]. Fig. 1 shows an example of such a separation using the oligosaccharide alditols in Table 1. The afore-mentioned generalizations concerning the number of sialic acids and the increased retention by N-glycolyl group are operative in these cases also.

9. Newer columns and instrumentation

Although the pulsed amperometric detector (PAD-II) was first commercialized by Dionex, other manufacturers have also developed similar

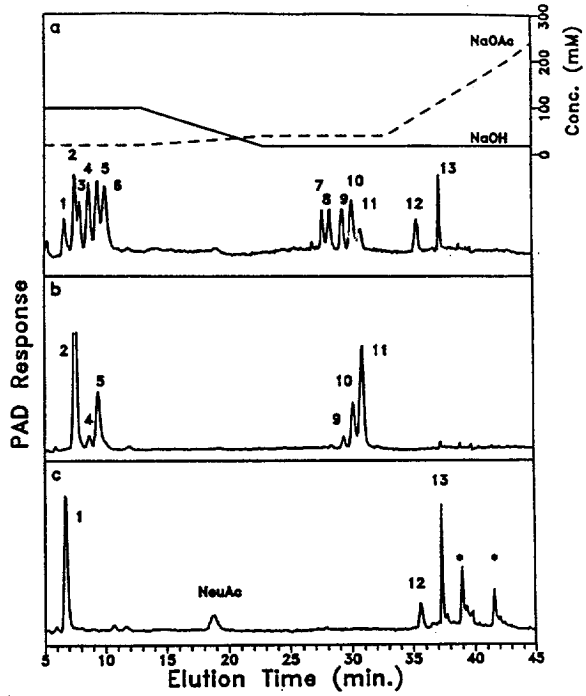

Fig. 1. HPAEC of sialyl oligosaccharide-alditols of O-glycosides. (a) The standard oligosaccharides; (b) the O-linked oligosaccharide mixture from bovine submaxillary mucin (3.5 μg); and (c) the O-linked oligosaccharide mixture from bovine fetuin (20 μg). The eluent used was: 0–13 min, 100 m*M* NaOH/20 m*M* NaOAc; 13–23 min, 100–18 m*M* NaOH/20–40 m*M* NaOAc; 23–33 min, 18 m*M* NaOH/40 m*M* NaOAc; 33–43 min, 18 m*M* NaOH/40–200 m*M* NaOAc; 43–48 min, 18 m*M* NaOH/200–300 m*M* NaOAc (shown in the top of the Panel a). The rest of the chromatographic conditions are described in the text. Structural assignment of the peaks is shown in Table 1. The peaks with asterisks in the Panel c were derived from N-linked oligosaccharides of bovine fetuin [22].

electrochemical detectors. A pulsed electrochemical detector (PED, Dionex, Inc.) which can also be used for conductivity measurement has been in use for some time, and a more recent electrochemical detector (ED40, Dionex, Inc.) offers a higher sensitivity [24].

As mentioned above, the pellicular resin columns, such as CarboPac PA-1, are the indispensible part of HPAEC. In recent years, newer columns have been introduced. There is a column to improve the resolution of higher oligosaccharides (NucleoPac PA-100) [25] and a column more suitable for reduced oligosaccharides

Table 1
Structure and relative PAD response of standard sialyl oligosaccharide-alditols

Number	Structure	Relative PAD response[a]
1	NeuAcα3Galβ3 / GalNAc-ol	0.92
2	NeuAcα6 \ GalNAc-ol	1.0
3	NeuAcα6 \ GalNAc-ol; Galβ3 /	1.3
4	NeuAcα6 \ GalNAc-ol; GalNAcα3 /	1.1
5	NeuAcα6 \ GalNAc-ol; GlcNAcβ3 /	1.5
6	NeuAcα6 \ GalNAc-ol; Galβ4GlcNAcβ3 /	Not determined
7	NeuGcα6 \ GalNAc-ol; Fucα2Galβ4GlcNAcβ3 /	Not determined
8	NeuGcα6 \ GalNAc-ol; Galβ4GlcNAcβ3 /	Not determined
9	NeuGcα6 \ GalNAc-ol; GalNAcα3 /	1.2
10	NeuGcα6 \ GalNAc-ol; GlcNAcβ3 /	1.6
11	NeuGcα6 \ GalNAc-ol	1.4
12	NeuAcα3Galβ4GlcNAcβ6 \ GalNAc-ol; NeuAcα3Galβ3 /	0.99
13	NeuAcα6 \ GalNAc-ol; NeuAcα3Galβ3 /	0.68

[a] Relative to 2 (NeuAcα6GalNAc-ol).

(CarboPac MA-1). Interestingly, the MA-1 column can also be used to separate reducing monosaccharides, but the elution sequence (in the order of Fuc, Man, Gal, Xyl) is different from that obtained from the PA-1 column (in the order of Fuc, Gal, Xyl, Man). There is no simple explanation for this phenomenon.

Optimization of amperometric pulsing conditions of the PAD for carbohydrates has been reported [26,27]. PAD can also be used to detect

aliphatic alcohols, and optimal wave forms using Pt and Au electrodes have been investigated [28].

10. Different pushing reagent

The eluents for HPAEC have been mostly NaOH of appropriate concentrations with and without NaOAc (the "pushing agent"). This is based on the preliminary results [29], which compared acetate, carbonate, and sulfate as "pushing agents." The combination of NaOH and NaOAc has been working well, although occasionally, non-alkaline conditions have been used in analyses of acidic oligosaccharides or polysaccharides [9,30].

The anion affinity for strong anion-exchange resins is known to be hydroxide < nitrate < sulfate. Wong and Jane [31] re-examined the effect of the "pushing agents", and concluded that nitrate actually gives a higher resolution and greater sensitivity when used with a CarboPac PA-1 column and PAD detector. The gain on the sensitivity when the column was eluted with nitrate was ca. 3-fold over that with acetate (Fig. 2). There was some improvement in resolution also. Although their work was aimed at the glucose-oligosaccharides generated by "enzymatic debranching" of starches, it should also be applicable for separation and detection of other types of oligosaccharides. The added advantage may be that by the use of nitrate, a lower NaOH concentration may be used for elution, which reduces the chances of epimerization and degradation when alkali-sensitive terminal sugars are present in the oligosaccharide. However, one predictable disadvantage of using nitrate as "pushing agent" is that desalting now requires de-anionization as well as de-cationization, because nitric acid (produced by decationization) must be removed befor evaporation of the oligosaccharide solutions to avoid alteration of the isolated products..

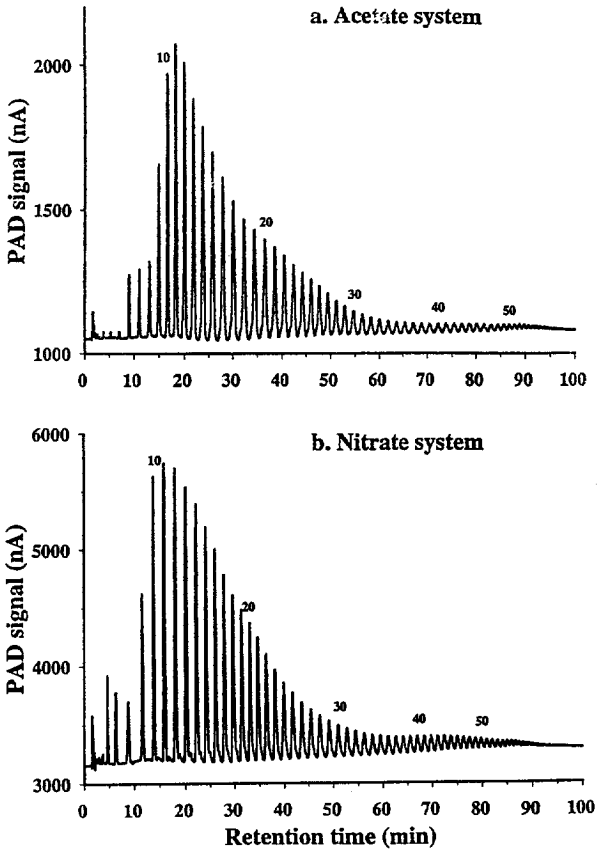

Fig. 2. Comparison of two "pushing agents" in analysis of homologous Glc-oligosaccharides by HPAEC. (a) Acetate system, (b) nitrate system [31].

11. Sulfated and phosphorylated mono- and oligosaccharides

11.1. Sulfates

Positional isomers of Gal and GlcNAc sulfates can be separated on a CarboPac PA-1 column. This was the basis of determining the position of sulfation in GlyCAM [11]. A similar separation of isomeric sulfoethylated glucoses was accomplished with the PA-1 column for characterization of the modified polysaccharides [32]. Alditols from sulfated di- and oligosaccharides derived from glycosaminoglycans by enzymatic action were separated by the PA-1 column [20]. Although alkaline eluents were used for these oligosaccharide alditols, detection was by means

of UV absorption rather than PAD, exploiting the absorbance of the 4,5-unsaturation generated by the enzymatic action. Sulfated sialyl oligosaccharides were isolated from tracheobronchial mucous glycoproteins with the aid of HPAEC [33].

11.2. Phosphate esters

Separation of positional isomers of phosphates has been mentioned above [9,10]. Sugar nucleotides, nucleotides and sugar phosphates were separated on an OmniPac PAX-100 (Fig. 3), and the technique was used to assay UDP-4′-glucose epimerase activity of cell-free extracts from *Neisseria meningitidis* [12].

12. Use of neutral or lower pHs

Although HPAEC mostly uses strongly alkaline eluents, there are some occasions when neutral or low pH are preferred. For example, in separation of sialylated and phosphorylated oligosaccharides, use of low pH eluents can provide better resolution than higher pHs [9]. It was reported that high pH conditions were required for the optimum separation of fetuin oligosaccharides, while low pH significantly improved resolution of oligosaccharides obtained from orosomucoid, human chorionic gonadotropin, platelet-derived growth factor, and kallikrein [30]. A scheme in which only 0.5 m*M* NaOH and 3% socium acetate (3% AcOH titrated with 50% NaOH to pH 5.5) were used for gradient generation was shown to be useful for separation of mono- as well as oligosaccharides [15].

13. Desalting of separated compounds

Because eluents of high salt concentration are used in HPAEC, desalting of the separated oligosaccharides is always troublesome. When the salt content is not too high, the in-line membrane desalting device (AMMS) is satisfactory [21], but for high oligosaccharides eluted with higher salt concentration, this may not be sufficient for complete desalting. The often practiced methods for desalting the HPAEC samples containing high levels of salts are dialysis, ion-exchange, and gel filtration.

In the dialysis process, the choice of the "Molecular weight cut off" (MWCO) range as well as the time for dialysis is quite important. It should be remembered that the "Molecular

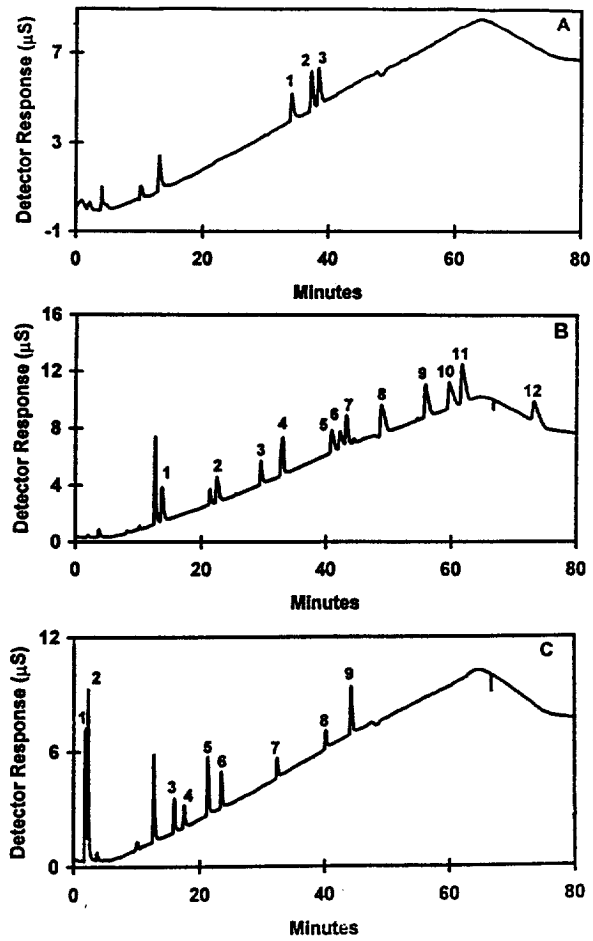

Fig. 3. Analysis of sugar nucleotides, nucleotides, and sugar phosphates by HPAEC–CD. A: UDP-Gal and UDP-Glc (1), UDP-GalNAc (2), and UDP-GlcNAc (3); B: CMP (1), AMP (2), CDP (3), UMP (4), ADP (5). GMP (6), CTP (7), UDP (8), ATP (9), GDP (10). UTP (11), and GTP (12); C: lactate (1), pyruvate (2), Glc-6-phosphate (3), Fru-6-phosphate (4), Pi (5), 3-phosphoglycerate (6), Glc-1,6-bis-phosphate (7), Fru-1,6-bis-phosphate (8), and 2,3-*bis*-phosphoglycerate (9) [12].

weight cut off" range is provided by the manufacturers only as a guide and not to be taken literally. Regardless of the nominal MWCO range, the prevention of leakage of the desired compound outweighs the necessity of complete desalting by prolonged dialysis.

Some oligosaccharides are eluted with such high salt content that direct lyophilization (after neutralization with acetic acid) produces too thick a syrup to be directly applied onto a gel filtration column. If the samples are diluted to a suitable viscosity for gel filtration, either multiple gel filtration operation or an unrealistically large column is required, either of which would lead to an excessively large eluate volume for a minute quantity of the sample, and thus increase the chance of contamination. The best compromise is to use a low MWCO dialysis tubing to dialyze for a short period of time, so that the sample can be lyophilized for application to a gel filtration column [34].

An alternative approach is to use ion-exchange resins. If the eluate contains only acid-tolerant compounds, the eluate can be directly treated with a cation-exchange resin (e.g., Dowex AG50) to completely remove sodium ion. Of course, the eluate would contain acetic acid, if NaOAc is also present in the eluate. However, since it is volatile, the samples can be evaporated or lyophilized. If the sample is extremely acid sensitive, and the presence of acetic acid is objectionable even during lyophilization, sodium ion can be exchanged for ammonium ion to maintain general neutrality of the sample. This is easily accomplished by treating the samples with a strong cation-exchange resin in ammonium form. This would generate ammonium acetate, which can be removed by repeated lyophilization.

14. Combination with other analytical tools

HPAEC and PAD, each an independent component, can be used with other instruments or components to gain additional usefulness. A PAD-II detector was successfully used with reversed-phase HPLC within a limited concentration of acetonitrile [35] and methanol [8]. The subsequent advent of the PED detector further expanded compatibility with organic solvent. More recently PAD-II was used to analyzed effluents of a graphitized carbon column (GCC) [36]. An experimental amperometric detector was also used in conjunction with CZE [37].

The excellent resolution of the MicroBeads columns is ideal for use with other instrumentation. Most obviously, oligosaccharides separated by HPAEC can be examined by NMR after desalting. This approach has been very effective in the determination of new oligosaccharide structures [38,39]. Examination of radiolabelled sulfated sugars from GlyCAM for identification of positional isomers were accomplished with a CarboPac PA-1 column [11]. Likewise, the presence of O-GlcNAc in some types of adenovirus was demonstrated by separation of radiolabeled sugars by HPAEC [40]. Other applications include characterization of glycopeptide peaks separated by reversed-phase HPLC [41] and monosaccharide and oligosaccharide analysis of glycoproteins transferred to PVDF membranes after SDS-PAGE [42]. The same methodology has also been applied to analysis of O-glycosides of isoelectric focusing-separated and blotted granulocyte colony-stimulating factor glycoforms [43]. After separation of oligosaccharides by HPAEC, they can be derivatized for analyses by other chromatographic or electrophoretic methods. Oligosaccharides from the erythropoietin produced in Chinese hamster ovary (CHO) cells were isolated by HPAEC and derivatized with 2-aminopyridine for analysis by reversed-phase HPLC [34].

There are a number of examples for using mass spectroscopy (MS) in conjunction with HPAEC for structural characterization. MS is quite suitable for use with HPAEC because only very small quantities are required, HPAEC was used to separate oligosaccharides which are analyzed by various forms of mass spectroscopy [18,38,44–49]. Some forms of MS are suited for on-line analysis of chromatographic effluent (e.g., ion-spray MS for reversed-phase HPLC of PA-oligosaccharides [50].) Similarly, HPAEC

equipped with the on-line desalting device (AMMS) was adapted for LC–MS [51].

Separation of sugar nucleotides, nucleotides, and sugar phosphates was accomplished with an OmniPac PAX-100 column with a conductivity detector (CD) rather than with PAD [12]. Presumably the same setup can be used for analysis of sulfated oligosaccharides or oligosaccharides containing uronic acids.

15. Applications

15.1. Analysis of enzymatic reaction products

Trimming of an oligosaccharide with exoglycosidases is a frequently used technique in the analysis of PA-derivatized oligosaccharides [13]. This approach also works well with analysis using HPAEC [15]. Moreover, we have used HPAEC to monitor transsialylation carried out by *Trypanosoma cruzii* [52] and transglycosylation of high mannose-type oligosaccharides by an *Arthrobacter* endo-β-N-acetyl-glucosaminidase (Fan et al. [67]).

HPAEC is especially suited for the analyses of the time course of enzymatic reactions, because: (i) products as well as starting materials can be measured without derivatization, (ii) elution conditions can be adjusted so that the results can be seen within a short time, (iii) detection is sensitive and would consume only small amounts of samples, (iv) the usually alkaline condition of the elution would stop most enzymatic reactions as soon as the sample is injected on the column, and (v) most biological materials are quite soluble in alkaline media and thus the need for removal of precipitates which often form when acid is used to stop the reaction is eliminated.

15.2. Examination of hydrolytic conditions

Ease of monosaccharide analysis by HPAEC–PAD enabled reexamination of optimum hydrolytic conditions for sugar component analyses. Hydrolysis of uronic acid-containing materials with trifluoroacetic acid (TFA) alone was found insufficient unless it is preceded by methanolysis [53]. Similarly, bacterial polysaccharides containing phosphodiester were found to be best hydrolyzed first with HF to cleave phosphodiester before hydrolysis with TFA [54]. Comparison of hydrolytic conditions for N-linked oligosaccharides in glycoproteins has been carried out with the aid of HPAEC–PAD, and the conditions of 2 *M* TFA at 100° for 4–6 h for neutral sugars and 4 *M* HCl at 100° for 6-8 h for amino sugars are found to be optimal [55].

15.3. Other applications

Shortly after its introduction, HPAEC–PAD was adopted very quickly by the biotechnology industry, which must deal with the carbohydrate portions of glycoprotein products. It is thus no surprise that there has been abundance of data from the analyses of "glyco-drugs". HPAEC–PAD perhaps is the simplest and most efficient method for quality control of glyco-drug production. Early examples of such analyses came from t-PA [45] and EPO [34], but there have been many others [39,56,57].

Wide availability of HPAEC–PAD instruments resulted in many other interesting applications, which take advantage of its speed and sensitivity. Lysosomal storage diseases, in which glycoconjugates are accumulated in the lysosomes because of the lack of certain hydrolases, were screened with the aid of HPAEC–PAD [58]. Quantitative carbohydrate analyses of the tectorial and otoconial membranes of the guinea pig were performed by this methodology [59]. Detection of orange juice adulteration with beet medium invert sugar was made possible by using HPAEC–PAD [60]. A fine analysis of steric divergence of tritium-labelling during the reduction of a wide variety of reducing sugars was accomplished with the aid of the superb resolution of CarboPac PA-1 column [61]. Analysis of 1- or 2-deuterated D-glucoses was reported [62].

16. Database and prediction of retention time

For peptides or nucleotides which have no branched structures, there are established sequencing methodologies. In these cases, the

problem is simply and literally to determine the sequence of linear arrangement of monomeric units. Unfortunately for carbohydrates, there are as many as 8 modes of adjoining two monomeric units (e.g., aldohexose) in a defined sequence, if they are assumed to be in either pyranose or furanose forms only. If the pyranose/furanose isomerism as well as branching is taken into account, the possibilities are enormous. Thus, the "sequencing" in the same sense as peptide sequencing or nucleotide sequencing does not exist for carbohydrates. While NMR measurement of a pure oligosaccharide, when available in sufficient quantities, can often yield sufficient information to deduce the entire structure, analyses of oligosaccharides by separation methods generally relies on comparison of unknown peaks with those of established reference compounds.

To analyze a compound or a mixture of compounds by any separation method (HPLC, CZE, etc.) is in reality to compare the unkown compounds with the known compounds directly or indirectly. For example, in amino acid analysis by cation-exchange chromatography or by reversed-phase HPLC after pre-column derivatization, all the amino acids expected to be present are well separated, and thus the peaks from any unknown samples can be assigned to one of the peaks in the standard mixture. This is also the case when the sugar composition of a glycoprotein is analyzed. The expected component sugars, e.g., mannose, galactose, glucosamine, and fucose, are well separated by HPAEC and thus each of the peaks in the hydrolysate can be assigned to one of the standard sugars (release of sialic acids require a different set of condition and a different separation condition.) However, when oligosaccharides are analyzed and their structural identities are being sought, the situation gets more complicated.

Unfortunately, there are only a limited number of pure reference oligosaccharides available commercially, and when they are available, their costs are often prohibitive for average research laboratories. Thus there are needs for establishing a method for predicting the chromatographic data (i.e., elution time) for an oligosaccharide of known structure and conversely for deducing the structure of an unknown oligosaccharide from the chromatographic data. This approach has considerable success in reversed-phase HPLC of PA-derivatized oligosaccharides [17,63], and the reliability of the method has been proven by other methods. An attempt has been made in this regard for HPAEC earlier [64]. More recently, an empirical approach was proposed for N-glycan mapping, which uses two optimized linear gradients for sialylated and asialo-N-glycans [65]. It was reported that the retention times of individual oligosacharide, prepared and measured on different days, were shown to be highly reproducible after the "validation" process. Using internal standards, $(Neu5Ac)_1$, $(Neu5Ac)_2$, $(Neu5Ac)_3$, and Neu5Gc, a database was established so that structural analysis of carbohydrate can be accomplished by mere comparison of retention times.

17. Perspectives

The use of HPAEC is now widely accepted for carbohydrate analysis, and new applications will continue to appear. However, higher sensitivity comparable to those available in peptides and nucleotides would be continually demanded. The new ED40 detector is a step toward this direction. Another improvement that would be desirable is the selectivity of the detector. The interference by peptidic and other non-carbohydrate materials should be eliminated or suppressed.

Generalization on the behavior of carbohydrates on a given column may not apply to a different column. The differences in the elution sequences of monosaccharides between the PA-1 and the MA-1 columns is a case in point. Different relative peak positions of monosaccharides obtained by the two different columns can be advantageous in some applications. This type of "multi-dimensional" approach has been successfully used in HPLC of PA-oligosaccharides [17,63,66]. Additional dimension for HPAEC may also come from other separation method such as capillary zone electrophoresis (CZE).

Undoubtedly, HPAEC will receive keen competition from CZE. CZE can be more sensi-

tive and may require less time for analysis. The shortcomings of CZE, such as requiring attachment of chromo- or fluorophores can be partially overcome by monitoring at low (195 nm) wavelength. Moreover, analysis of carbohydrates separated at pH 13 and measured with amperometric detector using copper electrode shows considerable promise [37].

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