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Journal of Chromatography A, 720 (1996) 173–182

JOURNAL OF
CHROMATOGRAPHY A

Review

Precolumn derivatization for chromatographic and electrophoretic analyses of carbohydrates

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Abstract

Sensitive detection and high chromatographic resolution are required for analysing the structures of oligosaccharides available in only limited amounts. Precolumn derivatization is one of the most suitable methods for this purpose as it can compensate for the shortcomings of the intrinsic nature of oligosaccharides. Recently published methods of precolumn derivatization are reviewed and advantages and problems are discussed.

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1. Introduction

Uncovering a new molecule is often the first step in a new field, and developing a new analytical method with an original principle is one of the approaches for this purpose. Recent progress in the chemistry and biochemistry of oligosaccharides, especially those liberated from glycoconjugates, has been largely due to the development of new analytical methods. However, analysis of the structures of oligosaccharides with higher precision and sensitivity is still required; a lack of sensitivity in any one of the procedures included in their structural determi-

nation, such as liberation, purification and structure elucidation, means this high sensitivity requirement will not be met.

One of the main problems in the chemical analysis of sugar structures is detecting the positions of the oligosaccharides. A highly sensitive means of oligosaccharide detection is particularly required because the amounts liberated from naturally occurring glycoproteins are often limited. Oligosaccharides, however, lack chromophores that are sensitive to optical detectors. UV, refractive index and pulse amperometric detection are not sufficiently sensitive for the above purpose.

A second problem is that sugars, such as glucose, galactose, mannose and N-acetylglucosamine, have similar structures and, in contrast to amino acids and proteins, oligosaccharides liberated from glycoconjugates are usually a mixture of several oligosaccharides with similar structures (microheterogeneity). Hence the separation of the oligosaccharides with high resolution can provide a sensitive structural analysis.

Sensitive detection and high resolution are the minimum requirements. Spectrophotometric analysis (postcolumn labelling) has been used, but it is not suited to high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). In these circumstances, precolumn derivatization could compensate for the lack of intrinsic characteristics of oligosaccharides and is suitable for sensitive analysis, in addition to being appropriate for the collection of the purified oligosaccharides after separation, a procedure indispensable for structure analysis.

Although precolumn derivatization is one of the most suitable approaches to compensate for the above-mentioned shortcomings and to raise the signal-to-noise ratio, it requires strict chemical techniques that must fulfil several reaction conditions. The derivatization reaction must be well defined, quantitative and non-destructive to other parts of the oligosaccharide without desialylation, epimerization or β -elimination at the reducing end sugar residues. Favourably, the reaction is specific to oligosaccharides among other biological components such as amino acids, proteins and lipids. Excess reagents and by-products formed during the derivatization reaction must be easily removed. The chemical groups introduced must be stable and able to withstand the chemical conditions required for the analytical procedures used in the structure determination. The derivatives must give identical molar responses regardless of the sugar sequences; this characteristic is needed to determine molar ratios of unknown oligosaccharides present in a sample from peak areas on HPLC traces.

The chemical groups that provide clues for the derivatization of oligosaccharides are a carbonyl group (aldehyde and ketone) of the reducing end

sugar residue, a hydroxyl group and a free amino group. Most oligosaccharides have a single reducing end, which is the major point for tags. Fluorescence, ultraviolet absorption, charge, biotin and radioisotopes are considered suitable as tags.

The derivatization aimed at the analysis of the structures of oligosaccharides is tritium labelling at the carbonyl group of the reducing end sugar residue [1]. This method has been used for the highly sensitive analysis of chemical structures from glycoconjugates [2,3]; however, it is hardly appropriate for HPLC and CE analysis, although a flow-through counter is available. There have been reports on the Schiff bases of oligosaccharides [4,5], but these are not stable enough for the analysis of chemical structures.

The first attempt to derivatize oligosaccharides in the sense described above was by pyridylation, i.e., the tagging of 2-aminopyridine to oligosaccharides by a reductive amination reaction aimed at analysing 2-aminopyridine-derivatized oligosaccharides by a two-dimensional sugar map (1978) [6]. Initially, it was not possible to achieve good separations of the derivatives, although the fluorescent pyridylamino group introduced is highly sensitive to optical detectors. Later, however, superior separation was achieved by reversed-phase HPLC owing to the hydrophobicity of the tag, while most oligosaccharides are too hydrophilic for separation by reversed-phase HPLC. In addition to its usefulness for sensitive detection and high resolution, the tag has advantages for mass spectrometry and NMR spectroscopy. When the derivatives of oligosaccharides are purified and their structures elucidated, they are frequently used for biological analysis, where removal of the tag is often required. For this purpose, "convertible derivatization" was introduced by Kallin et al. [7] in 1988.

This analytical system, based on precolumn derivatization, is considered to be the best candidate for the highly sensitive and systematic analysis of sugar structures. A short overview of recently published works on precolumn derivatization for HPLC and CE is given in the following sections. Only a few reports have described

the necessary data for application to structure analysis, the precise yields of derivatization depending on the molecular size (monosaccharides to oligosaccharides such as N-linked sugar chains), the type of reducing end sugar residues, such as hexose, N-acetylhexosamine and ketose, and liberation of sialic acid residues during the derivatization reaction. There have also been only a few reports describing the molar sensitivity for derivatives of sugars such as hexose, N-acetylhexosamines and oligosaccharides. Among the reactions described below, carbonyl derivatization is specific to sugars, whereas hydroxyl group- and amino group-based reactions derivatize other biological products such as amino acids that are concomitantly present in a sample. The detection limits of the derivatives depend on the apparatus used and the intrinsic sensitivity of the chromogens introduced. A ketose such as fructose has seldom been tested for derivatization.

The reader should also refer to other reviews published previously [8–11].

2. Derivatization by reductive amination

Tagging of the reducing end carbonyl group (aldehyde and ketone) with an aromatic compound having a primary amino group by means of reductive amination is the most popular method used for the analysis of oligosaccharides. Once the tag has been introduced, the derivative is stable against alkaline and acidic solutions; strongly alkaline media can therefore be used as eluents, while free oligosaccharides may degrade. The principle was first realized by using fluorescent 2-aminopyridine (PA) in 1978 [6]. Using the same principle, more than fifteen derivatives with ultraviolet absorption or fluorescence have subsequently been reported (Fig. 1).

PA-oligosaccharides are stable under various conditions of structure elucidation, such as Smith degradation, methylation analysis, partial acetolysis and acid or alkali hydrolysis. The reaction is almost quantitative for monosaccharides to N-linked oligosaccharides without side reactions (less than a few percent) such as β -elimination, epimerization or desialylation

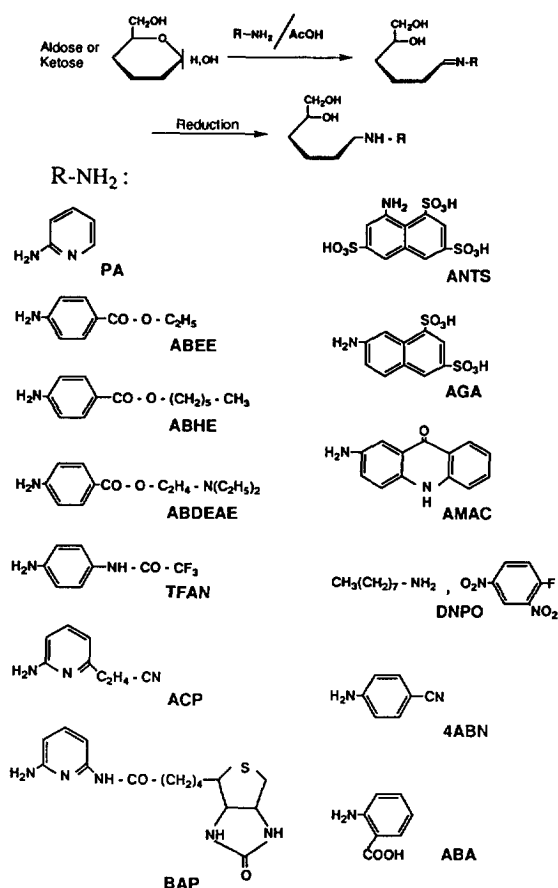


Fig. 1. Derivatization of reducing groups by reductive amination.

[12–15], and the derivatives have almost identical molar fluorescent intensities [13]. Desialylation was avoided by shortening the reaction time, raising the concentration of 2-aminopyridine or adding water to the reagent while quantitative yields were maintained [15]. The method has been used for determining the structure of oligosaccharides liberated from glycoproteins [16]. A two-dimensional sugar map [6,17–20] (see also the review by Takahashi in this volume [89]) made by size fractionation and reversed-phase HPLC is useful for assessing the structures of oligosaccharides in combination with exoglycosidase digestion, and a rule has been found correlating elution times and the chemical structures of the derivatives [17–19]. PA derivatives are used for the separation of

oligosaccharides by CE [21,22], for component sugar analysis [23–25], for structure analysis of oligosaccharides from glycoconjugates [26–29] and as substrates for enzymes [14,30–33]. The derivatives simplify NMR spectra [34] without the problem of anomeric configurations at the reducing end, and qualify the analysis by mass spectrometry. One picomole is sufficient for molecular mass determination by electrospray ionization mass spectrometry. When the capillary-skimmer voltage is raised, the mass spectrum drastically changes and gives ions of the pyridylaminated part of the fragments produced by cleavage at the glycosidic bonds (in-source fragmentation). These fragments can reconstitute the overall structure of the molecule [35,36]. The tagging with 2-aminopyridine provides homologues with a positive charge, which allows their analysis by electrophoresis. PA-maltooligosaccharides have been separated by capillary zone electrophoresis using a phosphate solution as the running electrolyte [37]. The pyridyl group of a PA-oligosaccharide can be removed for further derivatization (see Section 3).

The sugars are tagged with *p*-aminobenzoic acid ethyl ester (ABEE). The UV-sensitive detection of ABEE derivatives [38] has been used for the structure analysis of oligosaccharides released from glycoconjugates [39,40]. Fast atom bombardment mass spectrometry confirmed the molecular masses of the ABEE derivatives [38]. The greater the number of sugar units in an oligosaccharide, the more vigorous are the reaction conditions (longer reaction times, higher temperature, higher concentration) [38]. To avoid desialylation during ABEE derivatization, the concentration of acetic acid in the reaction mixture was reduced to 1/36 of that originally reported, and anhydrous conditions were used. The derivatization yield was then 80–90% for sialyllactose, and the extent of desialylation was less than 1% under the reaction conditions [39]. The reversed-phase HPLC separation of ABEE derivatives of neutral and amino sugars has been used for component sugar analysis. The peak-area ratios are almost identical among the sugars tested [41].

Fluorescent 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [42–45], 2-aminoacridone

(AMAC [45,46], and 7-amino-1,3-naphthalenedisulfonate (AGA) derivatives [47] have been combined with polyacrylamide gel electrophoresis for the convenient analysis of oligosaccharide structures. The trisulfonic acid ANTS was chosen as a single sulfonate cannot compensate sufficiently for the hydrophobicity of the reagent. The yield of the derivatization of glucose is more than 92%. The detection limit for ANTS-oligosaccharides is about 1 pmol per band (excitation at 370 nm, emission at 515 nm) and the cooled charge-coupled device has a limit of detection of about 0.2 pmol [42]. ANTS derivatives with a relatively high charge permit rapid separations for the larger oligosaccharides. ANTS-oligosaccharide can be electrophoretically resolved by discontinuous electrophoresis on high-density polyacrylamide gels based on size, charge and subtle structural characteristics such as linkages and monosaccharide composition. This technique has been applied to the qualitative analysis of small amounts of sugar chains from glycoproteins [43]. ANTS derivatives can also be separated by high-performance capillary electrophoresis. Baseline resolution of oligosaccharides with a degree of polymerization of ca. 70 is obtained within 15 min [48]. The use of ANTS derivatives has advantages in term of narrower peaks, greater resolution and shorter analysis times [49]. AMAC derivatives can be separated by polyacrylamide gel electrophoresis (Tris–borate buffer, pH 8.3), the gels being photographed on a UV light box (the detection limit was 0.6 pmol; 0.2 pmol with a charge-coupled device). Sialic acid showed three bands but it was not seen with sialylated oligosaccharides. As the AMAC derivatives have no charge at the pH used, they are suitable for the separation of neutral and acidic oligosaccharides and for the separation according to the structures rather than by size [46]. AMAC-N-linked oligosaccharides have been electrophoresed in high-density polyacrylamide gels [50], the derivatives being detected with excitation at 488 nm and emission at 520 nm. Micellar electrokinetic capillary chromatography (MECC) has been used for the separation of AMAC-oligosaccharides. Addition of β -cyclodextrin to the separation buffer permits

chiral discrimination; the interaction between β -cyclodextrin and taurodeoxycholate gives rise to an enhanced chiral separation [51]. The presence of a strongly charged sulfate group in AGA permits the use of a variety of buffers over a wide pH range. The AGA method does not require any specialized expensive equipment. Picomole amounts are readily detected by the human eye (emission maxima at 452 nm and excitation maxima at 314 nm). The yields for hexoses are 70–90%, GlcNAc 60–80%, oligosaccharides 50–80% and biantennary sugar chains 10%. Sialyllactose is coupled with AGA at a slightly elevated pH to prevent hydrolysis of the sensitive sialyl linkage [47].

n-Alkyl *p*-aminobenzoate (ABHE) [52], 4-aminobenzoic acid 2-diethylaminoethyl ester (ABDEAE) [53] and *N,N*-(2,4-dinitrophenyl)octylamine (DNPO) derivatives [54] are used especially for raising the sensitivity of analysis by mass spectrometry after purification by HPLC using UV absorbance detection. A hydrophobic tail was introduced into the oligosaccharides to promote surface activity within the matrix during liquid secondary ion mass spectrometry. *n*-Alkyl esters of *p*-aminobenzoic acid derivatives were tested to improve the sensitivity of the analysis by liquid secondary ion mass spectrometry, and *n*-octyl and *n*-hexyl *p*-aminobenzoate were found to be optimum considering the derivatization yield and mass spectral sensitivity [52]. Oligosaccharides derivatized with 4-aminobenzoic acid 2-diethylaminoethyl ester (ABDEA) have a basic tail provided by a 2-diethylamino group, which has a high proton affinity in the positive electrospray ionization mode. This leads to an improvement in sensitivity of more than 5000-fold over that with underivatized maltohexaose [53]. Oligosaccharide derivatization with an *N,N*-(2,4-dinitrophenyl)octylamine (DNPO) group also has a hydrophobic tail provided by an octyl group which enhances the surface activity and makes them amenable to separation by reversed-phase chromatography. The method gives a good yield of $[M-H]^-$ anions for the derivatized compounds, which are detectable at a level of about 1 pmol by fast atom bombardment mass spectrometry [54].

Sugars are derivatized by reductive amination

with 4-aminobenzonitrile (4ABN) and the derivatives are separated as their borate complexes by capillary zone electrophoresis or micellar electrokinetic chromatography. They are detected at the low femtomole level with UV detection at 285 and 305 nm [55].

Derivatization of glucosamine with 2-aminobenzoic acid (ABA, anthranilic acid) is accompanied by epimerization to mannosamine (>15%), but the epimerization can be reduced to <3%. The derivatives are detected by fluorescence (excitation at 230 nm and emission at 425 nm), and the detection limit is about 100 fmol. ABA does not react with sialic acid. The fluorescence intensity of the hexosamine is more than twice that of the neutral monosaccharides [56].

As bifunctional reagents which have two chemically active groups in the molecule, the following have been reported. The yield of trifluoroacetamidoaniline (TFAN) derivatization varied from 67 to 88% for monosaccharides to milk oligosaccharides. The derivatives were stable as lyophilized powders, but aqueous solutions gradually became discolored in contact with air [57]. TFAN derivatives are converted into isothiocyanate when treated with 25% aqueous ammonia followed by thiophosgene [57]. The cyano group of 2-amino-6-cyanoethylpyridine (ACP) is converted into a chemically active amino group by catalytic reduction without loss of fluorescence [58]. 2-Amino-(6-amidobiotinyl)pyridine (BAP) has a fluorescent pyridyl-amino group and a biotin residue which is a clue for biotin-(strepto)avidin technology, and is used for oligosaccharide derivatization [59]. Under the reaction conditions established, the yields for small oligosaccharides were 75–80% and for *N*-linked oligosaccharides 65–70%, and the extent of release of sialic acid was less than 5%. The detection limit was 50 fmol (excitation at 345 nm and emission at 400 nm). The biotinyl group can be used to recover the sugar chains from reaction mixtures. The high-affinity interaction of the biotinyl group with multivalent avidin or streptavidin can be used to create the functional equivalent of neoglycoproteins carrying multiple copies (theoretically four) of oligosaccharides of defined structure [59].

Using the same principle, derivatives have also

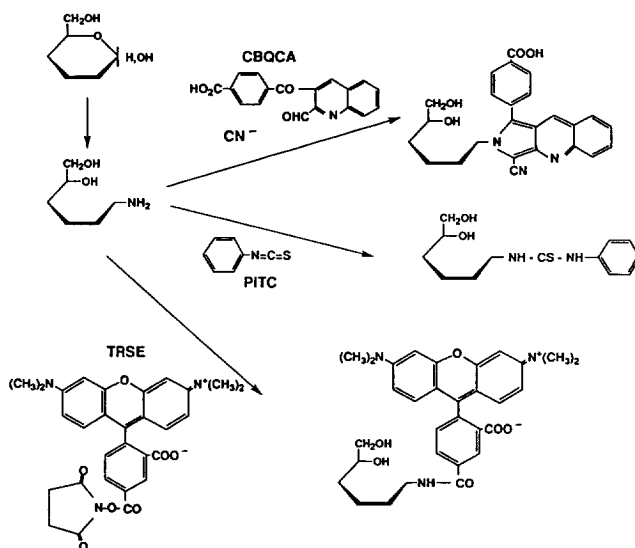


Fig. 2. Derivatization of oligosaccharides in a two-step reaction via the 1-amino-1-deoxy derivative.

been obtained in a two-step reaction (Fig. 2)—an intermediate, glycamine or 1-amino-1-deoxyalditol, is prepared with ammonium ion and NaBH_3CN or by catalytic reduction of the imine formed with benzylamine. After acid hydrolysis of oligosaccharides, neutral sugars are converted into glycamines, which are subsequently derivatized with phenyl isothiocyanate (PITC). The hexosamines are treated directly with PITC. The derivatives are separated by reversed-phase HPLC and determined down to the picomole level [60]. Glycamines are tagged with 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [61]. The minimum detectable amount is down to the low attomole level using capillary gel electrophoresis with laser-induced fluorescence detection (excitation at 457 and emission at 552 nm) [62]. The method was applied to hexose [61] and to amino sugars [63]. Glycamines are also tagged with 5-carboxy-tetramethylrhodamine succinimidyl ester (TRSE) [64,65]. 1-Amino-1-deoxyalditols and 2-amino-2-deoxyalditols are derivatized with TRSE, and the derivatives thus obtained are detected by fluorescence (excitation at 552 nm and emission at 570 nm). The detection limit was 60 molecules using capillary electrophoresis [65]. These reagents also react with amino groups of non-sugar

materials concomitantly present in the sample, hence the derivatations are less specific to the sugars than those using only reductive amination.

3. Convertible derivatization

Although the derivatization of oligosaccharides has advantages in detection and purification, the tag may not be necessary for further purposes after complex oligosaccharides have been purified and their structures elucidated. The tag can be removed in some cases, and the oligosaccharide part can be used for the subsequent experiments. This principle was first proposed by Kallin et al. [7] in 1988 using TFAN (Fig. 3). Treatment of the TFAN-lactose with aqueous hydrogen peroxide–acetic acid gives lactose in 76% yield, and with cerium(IV) ammonium nitrate the corresponding aminoalditol in 94% yield. An aqueous solution of TFAN-lactose became discoloured after 1 week in contact with air, but the derivative is stable enough for separation into its components by HPLC, and the reducing oligosaccharide is recovered in fair yields [7].

Pyridyl groups of PA-oligosaccharides can be

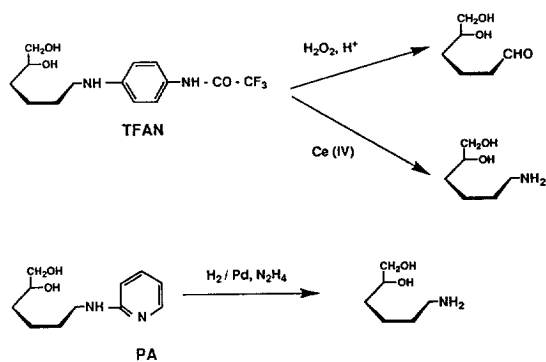


Fig. 3. Convertible derivatization.

removed, and 1-amino-1-deoxy glycitols are obtained quantitatively by catalytic reduction followed by mild hydrazine treatment [66]. The free amino groups thus formed can serve as linkage points for biotin, fluorescein, resins, etc.

4. Other tags at reducing end sugar residues with aromatic compounds

A glycosylamine or a Schiff base (imines) with a primary amine. The reaction is reversible and the mother oligosaccharide is easily recovered. The derivatives are thus somewhat unstable, and the problem of *syn/anti* isomers and anomers arises in the case of glycosylamine. Some glycosylamines are stable enough for purification by HPLC (Fig. 4).

Benzylglycosylamine (BA) (1956) [4] and fluorescent dansylhydrazine derivatives (1977) [5] have been reported. Dansyl hydrazones are detected with excitation at 370 nm and emission at 540 nm, and the detection limit is 2–5 pmol. Fructose reacts poorly with the reagent and elutes as two components. The molar response factors vary from 1.0 to 2.6 [67]. Fluorescent N-(2-pyridinyl) derivatives are also converted into the parent oligosaccharides by treatment with aqueous acetic acid [68]. Fluorescent Schiff bases formed with pentafluorobenzyl aminobenzoate (PFBAB) are also used for analysis. Under the reaction conditions established, free neuraminic acid was not detected when sialyllactose was derivatized. The yields were quantitative for

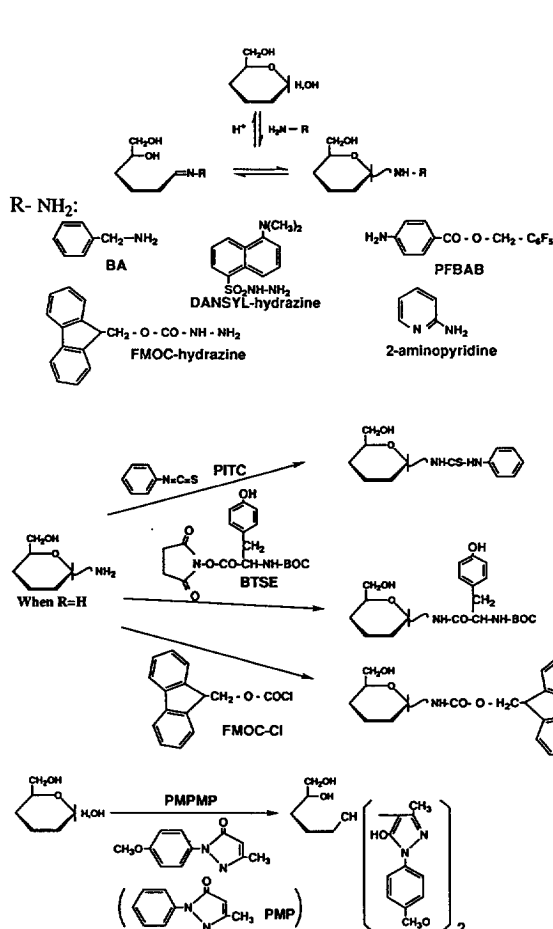


Fig. 4. Derivatization as a Schiff base or with pyrazolone derivatives.

small oligosaccharides, but for maltodextrin 80–90% and for N-linked oligosaccharides (high-mannose type) 65% (increased to 90% with longer reaction times, but sialyllactose showed a 6% loss of sialic acid). PFBAB-fucose (excitation at 350 nm and emission at 296 nm) was analysed by supercritical fluid chromatography–negative-ion chemical ionization mass spectrometry, and injection of 55 fmol provided an abundant signal for the main fragment [69]. Reducing oligosaccharides have been reacted with hydroxylamine followed by peracetylation, and the peracetylated aldonitriles or ketoximes were separated by HPLC. The derivatives are easy to prepare and stable. The detection limit

with UV detection is 3 μg per injection [70]. Fluorenylmethoxycarbonyl (FMOC)-hydrazones can be detected by fluorescence. The yield for mannose was 77% and for fucose 82%. Fructose exhibited the lowest response [71].

Reaction of lacto-N-tetraose with aqueous ammonium hydrogencarbonate gives the corresponding β -glycosylamine [72], which is reacted with FMOC-Cl to give an N-fluorenylmethoxycarbonyl β -glycosylamine. Detection of the derivatives is by absorbance at 256 nm. The yield for this two-step conversion is 66% and only the β -glycosidic derivative is formed. The derivatives are more stable than the TFAN derivatives towards air oxidation. The mother oligosaccharides can be recovered from the FMOC derivatives by treatment with aqueous ammonia followed by mild acidification with acetic acid. The yield was 93% for the lacto-N-tetraose derivative [73]. Glycosylamine obtained by the enzymatic hydrolysis of glycoprotein with peptide- N^4 -(N-acetyl- β -D-glucosaminyl)asparagine amidase F [74] is derivatized with PITC. Peptide- N^4 -(N-acetyl- β -D-glucosaminyl) asparagine amidase F with the optimum pH of 8.5 is a superior enzyme for generating glycosylamine because glycosylamine is more stable at the alkaline pH of the enzyme (pH 8.5) than at neutral to acidic pH. Less than 10% of the glycosylamines was converted into free oligosaccharides under the enzymatic reaction conditions employed [75]. Glycosylamine is also derivatized with *tert*-butoxycarbonyl-L-tyrosine N-hydroxysuccinimidyl ester (BTSE). The coupling yield was 80–90% for sialooligosaccharides. The products contain mainly a β -glycosylamide linkage and the proportion of the derivative having the α -configuration is less than 5%. The derivative can be radioiodinated to prepare oligosaccharide probes suitable for biological studies. The *tert*-butoxycarbonyl (Boc) group can be removed by treatment with trifluoroacetic acid without hydrolysing the glycosidic linkages. A single primary amine exposed allows the oligosaccharide to be coupled to other biological probes [76].

1-Phenyl-3-methyl-5-pyrazolone (PMP) reacts with reducing carbohydrates almost quantitatively under mild reaction conditions without

epimerization to yield strongly UV-absorbing (245 nm) and electrochemically sensitive derivatives. The detection limit is 1–0.1 pmol [77]. 1-(*p*-Methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP) is more reactive than PMP, the yields are almost quantitative and the detection sensitivity is about 50% higher than that of PMP-oligosaccharides. Sialyl residues are stable under the established reaction conditions and the PMPMP derivatives are stable at pH 4. However, collection of the HPLC eluate followed by evaporation causes partial decomposition of the product. The molar response for PMPMP-sialyllactose is almost the same as that of PMPMP-Glc [78].

5. Derivatization at hydroxyl or amino group

Ward and Pelter [79] separated sugars as benzoyl derivatives by HPLC in 1974. Derivatives of hexosamine and hexosaminitol with *o*-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol are analysed by reversed-phase HPLC. The derivatization is largely unaffected by the presence of salts. Glucosamine and galactosamine give two peaks due to the anomers. The detection limit for hexosamine is 1.6 pmol (excitation at 340 and emission at 450 nm). Picomole amounts of amino sugars can be conveniently and reliably analysed as their OPA-derivatized alcohols in the presence of amino acids [80]. Amino groups are also derivatized with phenyl isothiocyanate (PITC). The Waters PICO-TAG analysis procedure for amino acids is used to analyse hexosaminitol involved in the linkage to peptides [81]. The PTC derivatives of amino sugar alcohol show a single peak and are stable after derivatization, but those of GlcN and GalN show additional peaks. Both PTC-GlcN and PTC-GalN are quantitatively transformed into the conversion products within 2.5 h. The PTC derivatives thereof are detected at 254 nm, and less than 10 pmol of GlcN and GalN can be easily determined [82]. Amino groups are also derivatized with radiolabelled acetic anhydride, which is used for the analysis of O-linked oligosaccharides [83]. Since the amino groups of

amino sugar residues are usually acetylated, they must be set free before derivatization in this case.

6. Tritium labelling at reducing end sugar residues

Aldehydes and ketones of reducing oligosaccharides are reduced with sodium [³H]borohydride to glycol with the incorporation of tritium, as demonstrated by Conrad et al. [1] in 1966. The isotope label is stoichiometrically introduced, and widely used for component sugar analysis [84–86] and the structure determination of oligosaccharides from glycoconjugates [1–3,87]. A recent paper showed that borohydride reduction produced a characteristic and specific radioactivity, but the radioactivities on a molar basis varied [88].

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