1

GENERAL INTRODUCTION

Partially based on:
Oligosaccharide analysis by high-performance anion-exchange chromatography hyphenated to integrated pulsed amperometric detection and on-line ion-trap mass spectrometry

Cees Bruggink

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1.1 GLYCOBIOLOGY

Deoxyribonucleic acid (DNA) plays a central role in cell biology, because it is the biological information carrier required for the function of the cell. The flow of this information to generate other cellular molecules and metabolites is accomplished by translating parts from the DNA code into ribonucleic acid (RNA), and from RNA into the generation of proteins. Proteins and DNA alone are not the only essential classes of molecules necessary for a living cell, lipids and carbohydrates being two other classes of vast importance.

Carbohydrates can serve as energy sources, signaling molecules, or as construction components. Carbohydrates are often covalently bonded to other molecules like lipids and proteins forming glycoconjugates such as glycolipids and glycoproteins. From all proteins, more than 50% are glycosylated [1]. The glycan part of a glycoprotein is involved in many different biological processes such as protein folding, signaling, development of multicellular organisms, cell-cell communication and adhesion, cell-matrix interaction, fertilization, inflammation, and immune responses [2-9]. This list of examples is not complete but illustrates the importance of glycans. As a result of the disturbed production or degradation of glycoconjugates, a more or less severe disease can be developed [7,10].

It is essential to analyze the biochemical effects of disturbed glycoconjugate degradation in order to understand the molecular basis of the resulting human diseases. This includes the analysis of the glycan and glycoconjugates accumulated due to enzymatic defects, which is addressed in this thesis. In the following the methods applied in this thesis will be introduced (1.2.) followed by an overview on genetic, biochemical and clinical aspects of various defects in glycoconjugate degradation (1.3).

1.2 HIGH PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION (HPAEC-PAD)

Ion chromatography is a special form of high performance liquid chromatography for the analysis of a mixture of ionic species. For a good analysis of ions in a complex sample matrix, a separation prior to the detection is often required. This separation is needed because of interferences in the detection method due to chemical similarities of components. In these cases separation is an essential part of the analytical procedure. Another reason for applying a separation is the possibility to use nonselective detection techniques. Chromatographic techniques are particularly successful in combination of an efficient separation and instantaneous in-line detection [11,12]. Ion chromatography is routinely used for the analysis of inorganic as well as organic ions and polar components. Specifically, the analysis of carbohydrates by HPAEC-PAD is a very common and well accepted application for the characterization of free oligosaccharides which are derived from glycoproteins or glycolipids [13-15].

1.2.1 History of anion-exchange chromatography and detection of sugars

Already during decades anion-exchange chromatography has been used in different ways to separate and analyze carbohydrates. Initially Zill and colleagues made use of anionic sugar-borate complex formation and an anion-exchange resin in the borate form [16,17]. Various improvements of this method have been developed such as the use of a post column reaction prior to optical detection, gradient elution, and automation [18]. Such an automated system allowed the analysis of monosaccharides originating from glycoproteins [19]. The post column reaction with orcinol in hot concentrated sulfuric acid is very corrosive and aggressive and has been later substituted for a non-corrosive dye reaction for reducing sugars [20] or a fluorescence post column reaction [21]. These anion-exchange chromatography systems had various drawbacks such as a destructive detection method, slow elution resulting in broad peak shapes even in the case of shorter run times, and the lack of selectivity for oligosaccharides larger than dimers [22].

A big step forward in ion chromatography was the development of an agglomerated pellicular resin as stationary phase in combination with an eluent stripper column situated in front of the detector by Small *et al*. [23]. Ion chromatography was initially used for the separation of small ions and the stripper column was later named suppressor or desalter. Due to the faster exchange kinetics of this type of resin shorter run times with a better resolution were obtained in combination with chemically suppressed conductivity detection, a sensitive non-destructive detection method, without derivatization of the ions. In 1981 Hughes and Johnson reported about a sensitive electrochemical detection method for underivatized carbohydrates named pulsed amperometric detection [24].

A noble metal used as working electrode, such as platinum or gold, acts as electrochemical catalyst for oxidation reactions of carbohydrates. Consequently only a low potential is needed for detection

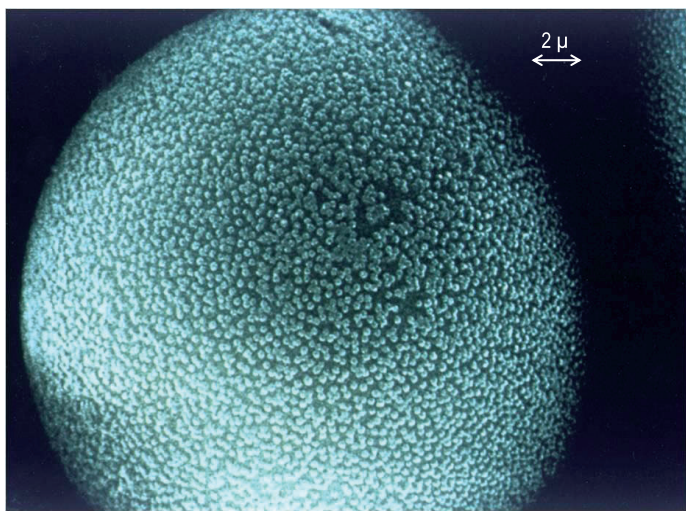

Figure 1-1. Scanning electron microscope (SEM) picture of an agglomerated pellicular resin. Reprinted with permission from [12].

and results in an optimal signal to noise ratio with a limit of detection in the pmol range. Parallel to the development of PAD carbohydrate separation was investigated employing strong anion-exchange columns packed with agglomerated pellicular resin (Figure 1-1) used with high pH eluents.

The combination of these detection and separation methods resulted in 1983 in a publication of Rocklin and Pohl describing the use of a low capacity anion-exchange resin with a high pH eluent for the separation and of carbohydrates followed by pulsed amperometric detection known as HPAEC-PAD [25].

1.2.2 Mechanism of separation

Anion-exchange chromatography can separate anionic carbohydrates that contain a sialic acid, a carboxylate, a sulfate, or a phosphate group [13,14]. Neutral carbohydrates are very weak acids and they dissociate in an eluent at a sufficiently elevated pH allowing their interaction with anion-exchange resins (Table 1-1).

From selectivity research with small anions it is known that analyte charge is the dominant retention factor, followed by the size of an ion [12]. The relevance of analyte charge is nicely illustrated in Figure 1-2 that shows the separation of phosphorylated sugars.

A monophosphorylated glucose (peak 4) elutes faster from the anion-exchanger than a diphosphorylated glucose (peak 13). In addition, at sufficient high pH neutral glycans will undergo dissociation resulting in negative charges. The influence of glycan size is illustrated in Figure 1-3 showing the separation of fructo-oligosaccharides where a higher degree of polymerization (DP) leads to longer retention times.

The separation of dihexose structural isomers in Figure 1-4 highlights the relevance of additional selectivity mechanisms other than those related to charge and size. This chromatogram shows separations of several dihexoses representing compositional isomers (i.e. peak numbers 2, 3, and 9), anomers and isomers differing in linkage positions (i.e. peak numbers 1, 4, 6, 7, and 9). Koizumi and colleagues [28] analyzed positional isomers of methyl ethers of D-glucose among other isomers with HPAEC-PAD and concluded that the reduction in retention time resulting from O-methylation follows the order of 1-OH > 4-OH ≥ 6-OH >3-OH >2-OH representing the difference in the acidity of the different alcohol groups. From this obtained data it is comprehensible that trehalose (Glc(α1-α1)Glc) elutes so early from the column (Figure 1-4) since both anomeric alcohol groups are blocked. Koizumi *et al*. also studied the retention of glucose disaccharides and compared the results with the findings obtained for O-methylated

Table 1-1. Dissociation constants of common carbohydrates in water at 25 °C [26].

Carbohydrate	**pK_a**
Fructose	12.03
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Dulcitol	13.43
Sorbitol	13.60
α-Methyl glucoside	13.71

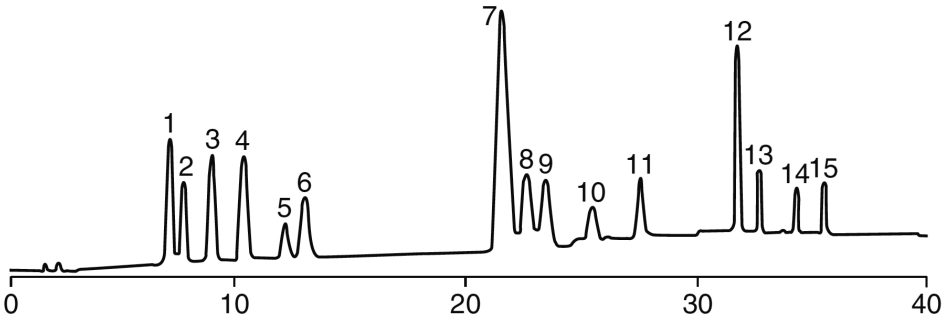

Figure 1-2. Separation of mono- and diphosphorylated monosaccharides with a CarboPac PA1 column using a sodium acetate gradient in 100 mM sodium hydroxide and PAD detection at a gold electrode. Peaks and amount (µg): (1) α-D-galactosamine-1-phosphate, 1.13; (2) α-D-glucosamine-1-phosphate, 0.45; (3) α-D-galactose-1-phosphate, 1.75; (4) α-D-glucose-1-phosphate, 1.75; (5) α-D-ribose-1-phosphate, 1.46; (6) β-D-glucose-1-phosphate, 1.75; (7) D-glucosamine-6-phosphate, 3.75; (8) D-galactose-6-phosphate, 2.04; (9) D-glucose-6-phosphate, 1.25; (10) D-fructose-1-phosphate, 0.96; (11) D-fructose-6-phosphate, 0.42; (12) α-D-glucuronic acid-1-phosphate, 3.08; (13) α-D-glucose-1,6-diphosphate, 1.06; (14) β-D-fructose-2,6-diphosphate, 0.92; (15) D-fructose-1,6-diphosphate, 0.92. Reprinted with permission from technical note 20 Thermo Fisher Scientific.

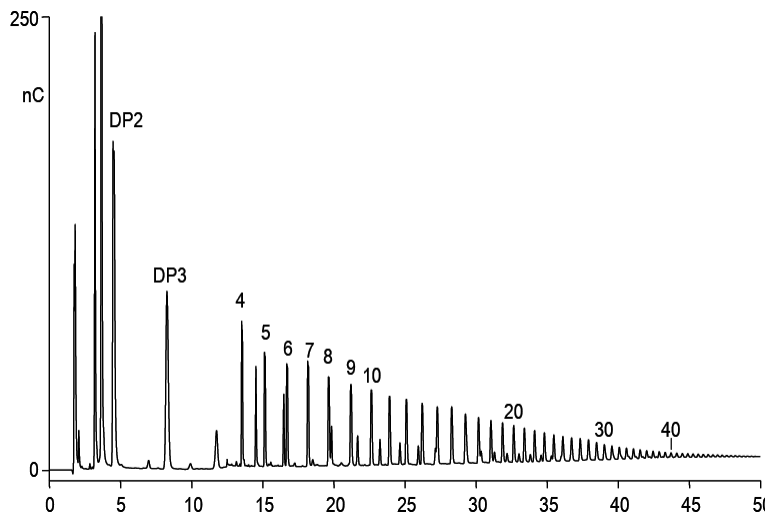

Figure 1-3. Separation of inulin with a CarboPac PA200 column using a sodium acetate gradient in 60 mM NaOH and PAD detection at a gold electrode. Reprinted with permission from [27].

glucoses showing that elution positions were somewhat effected by the configuration and hydrophobic interactions. In general, the α-isomer elutes faster from the column than the corresponding β-isomer with the exception of cellobiose and maltose (see Figure 1-4) [13,28].

A nice example of an isomeric *N*-linked oligosaccharide separation is shown in Figure 3-6, and an example of a baseline separation of two asialo-triantennary glycopeptides only differing

in the linkage of a galactose (either β1-3 or β1-4) has been published by Hardy *et al*. [29]. For a more in depth study of HPAEC oligosaccharide separations the following review articles are recommended: [13,14,29,30].

1.2.3 Detection by pulsed amperometric detection (PAD)

Underivatized carbohydrates can be sensitively detected with a gold working electrode, because aldehyde, ketone, and alcohol groups are oxidizable at 0.10 V versus a Ag/AgCl reference electrode [31]. Oxidation at this low potential is possible because gold serves as a catalyst in the oxidation reaction of the aforementioned groups [32]. To keep the gold electrode catalytically active, cleaning and activation potentials are applied after the detection potential [33]. All of these potentials are only applied for less than a second for the total sequence of potentials and their time periods being referred to as a waveform. A modern waveform provides two data points per second and is stable over a long period of time [33].

1.2.4 Mass spectrometric detection

To obtain more information from complex samples, particularly in those cases where standards are not available, mass spectrometric detection (MS) is advisable. To this end, the effluent of the column passing the amperometric detection cell can be fraction collected. These fractions need to be desalted prior to mass spectrometric analysis. Desalting can be performed off-line or more conveniently inserting a membrane device as an on-line desalter between the detector and the fraction collector [34,35]. Another approach is to analyze the fraction by HPLC-MS with the salt plug redirected to waste [36]. Direct hyphenation of MS to HPAEC using electrospray ionization (ESI) is particularly useful when the sample amount is limited, to identify glycans, and to unravel coeluting glycans in chromatograms of complex samples. The obtained signal intensity of carbohydrates is relatively low compared to that of peptides or proteins due to the low ionization efficiency of carbohydrates. Anionic carbohydrates can selectively be detected in deprotonated form with ESI-MS in the negative mode. Neutral carbohydrates easily form adducts with a proton or a metal ion so that they can be detected in the positive mode

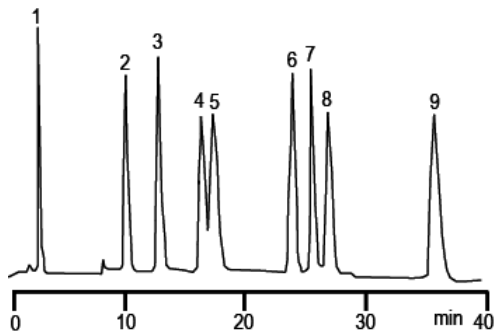

Figure 1-4. Separation of disaccharides with HPAEC-PAD. Peaks (1) Glc(α1,α1)Glc (trehalose); (2) Fru(β2,α1)Glc (sucrose); (3) Gal(β1,4)Glc (lactose); (4) Glc(α1,6)Glc (isomaltose); (5) Gal(α1,6)Glc (melibiose); (6) Glc(β1,6)Glc (gentiobiose); (7) Glc(β1,4)Glc (cellobiose); (8) Glc(α1,3)Fru (turanose); (9) Glc(α1,4)Glc (maltose). Reprinted with permission from technical note 20 version 1 Thermo Fisher Scientific.

[37,38]; see Table 1-2 for commonly used adduct ions. Mohr *et al*. [37] have studied the relative affinities of different alkali metal ions for carbohydrates, and have found that the affinity order is $Cs^+ > K^+ > Na^+ > Li^+ > H^+$. Notably, although cesium is generally most efficient at producing ions of oligosaccharides, it is inefficient in ionizing small carbohydrates [39].

Di- and trivalent metals are also capable of ionizing carbohydrates, but only singly charged quasimolecular ions are formed [40]. If the glycan contains fucose as constituent and proton adducts are formed, rearrangements can occur and the position of the fucose residue in the glycan may be changed upon tandem mass spectrometry [41-43] which may lead to structural misinterpretation. Lithium adducts are most sensitive for ionizing relatively small sugars. Carbohydrate lithium adducts easily undergo in source fragmentation [44]. While this is often viewed as a disadvantage because of signal intensity loss, it can be turned into an advantage when a single quadrupole MS detector is used to obtain more information about the eluting carbohydrate by efficient in source fragmentation [44]. Sodium adducts are more resistant to in source decay and are commonly used in MS detection. The stability of sodium adducts is such that with mild energy levels fragmentation can be induced with tandem MS allowing structural elucidation. The first piece of information to be obtained is the mass of the precursor / intact carbohydrate. From this mass and knowledge of the masses of contributing monosaccharides (e.g. hexoses have a mass increment of 162, *N*-acetylhexosamines of 203; Table 1-3) a composition can be obtained. Upon tandem MS the glycosidic cleavages provides the monosaccharide sequence, while cross-ring fragmentation can reveal linkage information, though the anomeric configuration (i.e. whether a linkage is α or β) can generally not be determined on the basis of MS spectra [45-47].

The broadly accepted nomenclature for assigning the fragmentation of carbohydrates was proposed by Domon and Costello [48] and is depicted in Figure 1-5. At the left side of the figure is the non-reducing end of the oligosaccharide, while at the right side the reducing end is situated. Abundant fragments are generated by cleavage at the glycosidic bonds. Fragments containing the non-reducing end are called B and C fragments, and Y and Z fragments contain the reducing end. A-ions are cross-ring fragments containing the non reducing part of the glycan, while X-ions are cross-ring fragments including the reducing part.

These cross-ring fragment ions are accompanied by a subscript indicating the position relative to the termini and a superscript in front indicating the cleavage positions in the ring, starting with 0 for the bond between the ring oxygen and the anomeric carbon with further counting in the clockwise direction (Figure 1-5). Linkage position information may be derived from cross-ring fragments. Table 1-4 lists mass losses observed depending on cross-ring fragmentation when tandem MS is used in the positive mode.

Table 1-2. Adduct ions and their use in the ESI (+) mode.

Adduct ion	Monoisotopic mass	Remarks
Hydrogen	1.01	Fucose rearrangement possible
Lithium	7.02	In source fragmentation easy to induce
Sodium	22.99	Commonly used
Potassium	38.96	Stable adduct
Cesium	132.91	Only for larger oligosaccharides

Table 1-3. Monoisotopic mass increments of some carbohydrates.

Carbohydrate residue	Residue Mass
Deoxyhexose	146.06
Hexose	162.05
Hexosamine	161.07
Uronic acid	176.03
N-acetylhexosamine	203.08
N-acetylneuraminic acid	291.10
N-glycolylneuraminic acid	307.09

Separation of carbohydrates with HPAEC is usually performed with sodium hydroxide in the eluent, and to elute oligosaccharides from the anion-exchange column a sodium acetate gradient is applied, while the hydroxide concentration is typically kept constant [49]. A mobile phase containing sodium hydroxide and sodium acetate is incompatible with the ESI interface of the mass spectrometer. To convert the eluent for on-line MS into a compatible fluid, a membrane based desalter is used. There are several types mentioned in the literature such as the carbohydrate membrane desalter (CMD) [34] and the anion self regenerating suppressor (ASRS) [44]. Such a membrane desalter can be considered as a cation-exchanger in the acidic form exchanging sodium ions for hydronium ions. The sodium hydroxide is converted into water while sodium acetate is converted into volatile acetic acid. The CMD and ASRS are continuously regenerated by electrolysis of water that is sometimes assisted with trifluoroacetic acid as proton donor to enhance the desalting capacity [34]. After desalting the eluent, a make-up solution is added via a T-connector to facilitate carbohydrate adduct - and spray formation, see Figure 1-6.

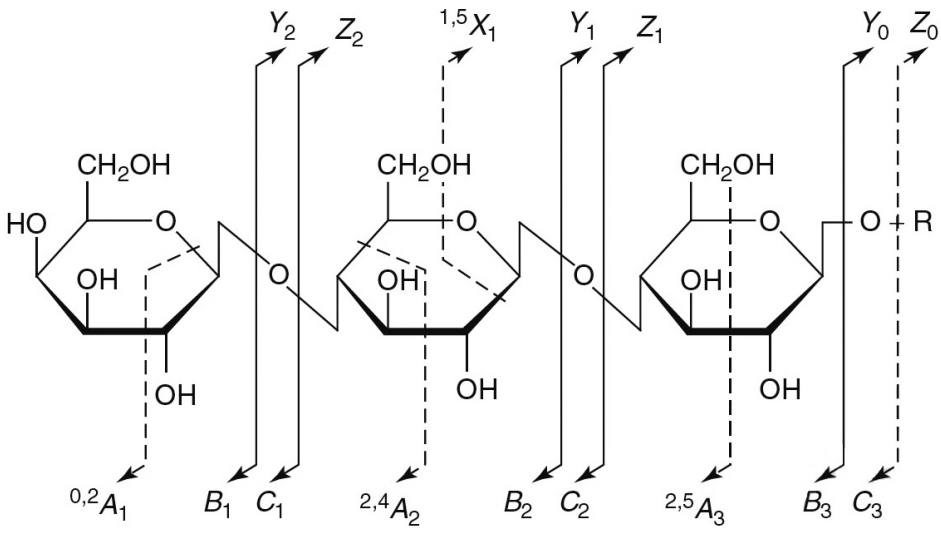

Figure 1-5. Glycan fragmentation types and their nomenclature as proposed by Domon and Costello. Reprinted with permission from [48].

Table 1-4. Mass difference relative to a C-type ion observed for sodium adducts of oligosaccharides upon A-type cross-ring cleavages of hexose (Hex) and *N*-acetylhexosamine (HexNAc) residues [45-47].

Monosaccharide		Linkage type			
Hex	**HexNAc**	**1 – 6**	**1 – 4**	**1 – 3**	**1 – 2**
-60	-101	$^{0,2}A$	$^{0,2}A$	-	-
-90	-131	$^{0,3}A$	-	-	-
-120	-161	$^{0,4}A$	$^{2,4}A$	-	$^{1,3}A$
-106	-147	$^{3,5}A$	$^{3,5}A$	-	-
-78		$^{0,2}A-H_2O$	-	-	$^{0,2}A-H_2O$

1.2.5 Application example: analytical-scale HPAEC-PAD-MS of released *N*-glycans

A mix of *N*-glycan standards representing structures such as they are typically found on therapeutic glycoproteins expressed in mammalian cell culture was analyzed by HPAEC-PAD-MS. The system was a Dionex ICS-3000 ion chromatograph consisting of a low pressure gradient pump, an isocratic pump, a chromatography detector module equipped with an electrochemical detector and cell outfitted with a gold working electrode and Ag/AgCl as reference electrode, injection valve and a dual zone temperature control, and a cooled autosampler. The MS was a Bruker HCT Ultra ion-trap equipped with an ESI interface. The separation was carried out on a CarboPac PA200 (3 x 250 mm) column from Dionex Corp. at 30 ºC and a flow rate of 360 µL/min. After the column a homemade PEEK splitter was installed which split 90 µL/min to the electrochemical cell and 270 µL/min to the desalter (ASRS-300 2 mm Dionex in the external water mode). The eluent leaving the desalter was combined with 90 µL/min 50% acetonitrile for MS detection in the positive mode detecting the glycans in the protonated form (Figure 1-6).

The resulting separation is shown in Figure 1-7. The different peaks are identified on the basis of MS and tandem MS spectra. In case of MS could only partly elucidate structure, structures were confirmed by running standard solutions of the different glycans. This application shows the successful hyphenation of on-line MS with HPAEC-PAD by adding a desalter in front of the ESI-MS/MS.

1.3 LYSOSOMAL CATABOLISM OF OLIGOSACCHARIDES

Lysosomes were described for the first time by de Duve in 1955 [50]. Unconnected lysosomes together form the endosomal-lysosomal system which is a characteristic space within a cell [50,51]. Within the lysosome an acidic environment is generated forming the digestion system of the cell. The digestion of oligomers to smaller products is mediated by hydrolases which generally have a pH optimum in the range of 3.5 to 5.0. Other proteins involved in lysosomal degradation processes are cofactors, activator proteins for glycosphingolipids, and carrier proteins that deliver catabolic products to the cytosol. The endosomal-lysosomal system has a central position in the economy of the cell [51].

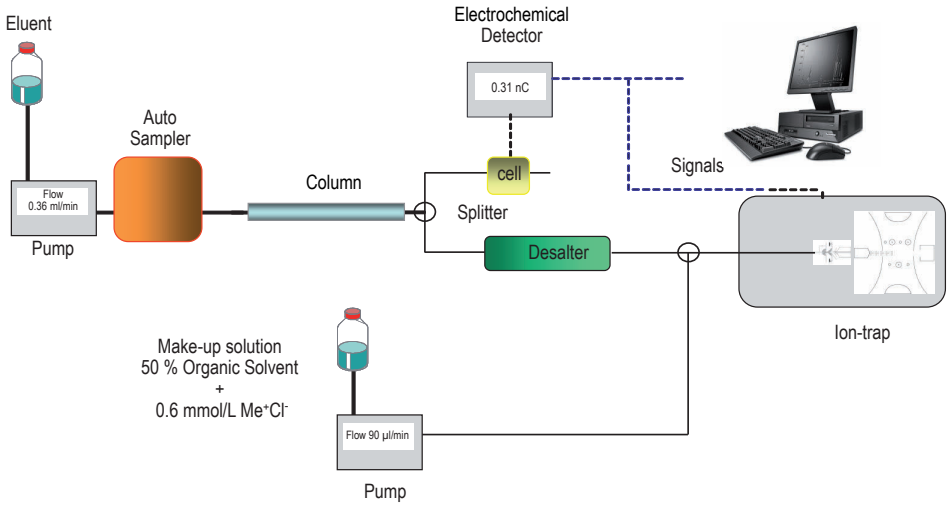

Figure 1-6. Instrumental setup for on-line HPAEC-PAD-MS. Eluent A was ultra pure water, B 200 mM NaOH, and C 400 mM NaOAc. The gradient was 0 – 5 min 50% A, 50% B, 0% C and from 5 – 65 min 0% A, 50% B, and 50% C. The PAD waveform applied to the gold working electrode was 0.00 – 0.20 s 0.10 V, 0.20 – 0.40 s 0.10 V (data collection), 0.41 – 0.42 s -2.00 V, 0.43 s 0.60 V, 0.44 – 0.50 s -0.10 V versus a Ag/AgCl reference electrode [33]. The figure is modified form [27] and reprinted with permission.

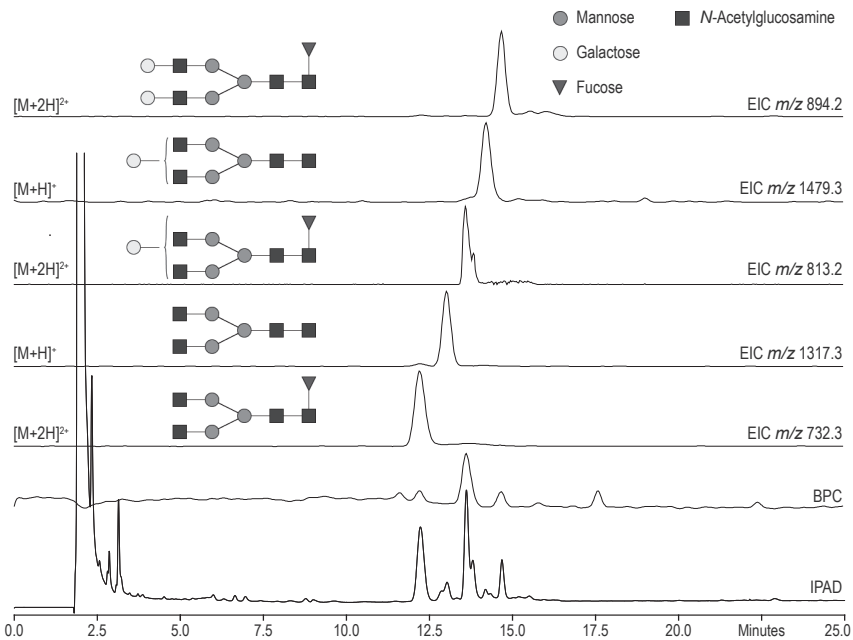

Figure 1-7. Separation of *N*-linked glycans with on-line HPAEC-PAD-MS using a CarboPac PA200 column. Reprinted with permission from [27].

1.3.1 Catabolism of Asn-linked glycoproteins in the human lysosome

There are three different types of oligosaccharides known to be linked via asparagine to human proteins: high mannose, hybrid type, and complex type N-glycans (Figure 1-8).

The acidic condition in the lysosome is required for the enzymatic activity of the lysosomal enzymes which have an acidic pH optimum and will also partially denature the protein substrates thereby making them more accessible for endo- and exohydrolases.

Complete breakdown of glycoconjugates to monomers is required to avoid lysosomal disorders that can become manifest when fragments as small as dimers are left undigested [52]. Catabolism of Asn-linked glycoproteins to monosaccharides and amino acids occurs in lysosomes. The first process happening in glycoprotein degradation is the hydrolysis of the protein backbone with endo- and exopeptidases until finally asparagine-linked glycans are left. Next, the degradation of Asn linked glycans happens in a highly ordered way (see Figure 1-9) [10]. First the removal of the core fucose (Fucα1→ 6GlcNAc) of complex type or hybrid type N-glycans and probably any peripheral fucose residues linked to the outer branches of the chain (Fuc α1 → 3GlcNAc) is performed by lysosomal α–fucosidase [10]. Aspartyl-*N*-acetyl-β-D-glucosaminidase then hydrolyses the GlcNAcβ-Asn bond followed by the removal of the reducing-end GlcNAc by endo-β-*N*-acetylglucosaminidase (chitobiase) as shown in primates and rats [52,53], leaving the oligosaccharide with only one GlcNAc at the reducing end. The

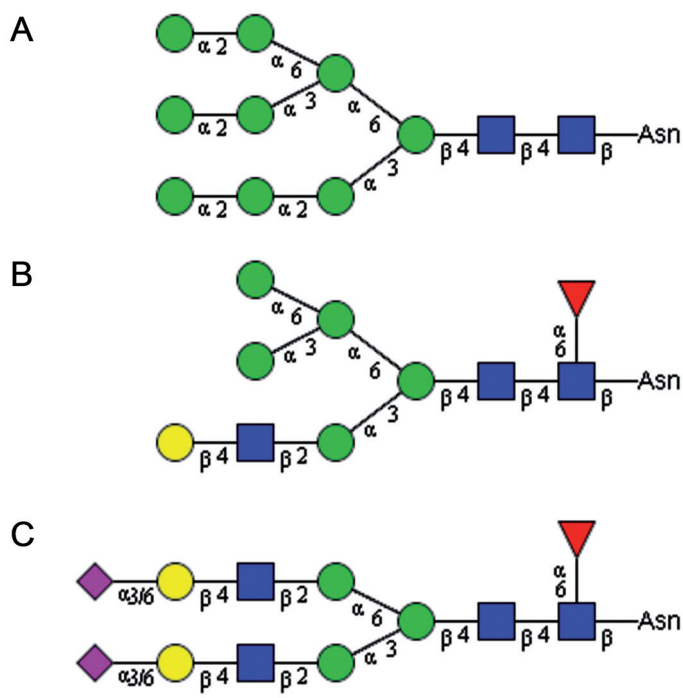

Figure 1-8. Three examples of asparagine linked oligosaccharides. High mannose type (A), hybrid type (B), complex type (C). Green circle mannose, yellow circle galactose, blue square *N*-acetylglucosamine, purple diamond *N*-acetylneuraminic acid, red triangle fucose.

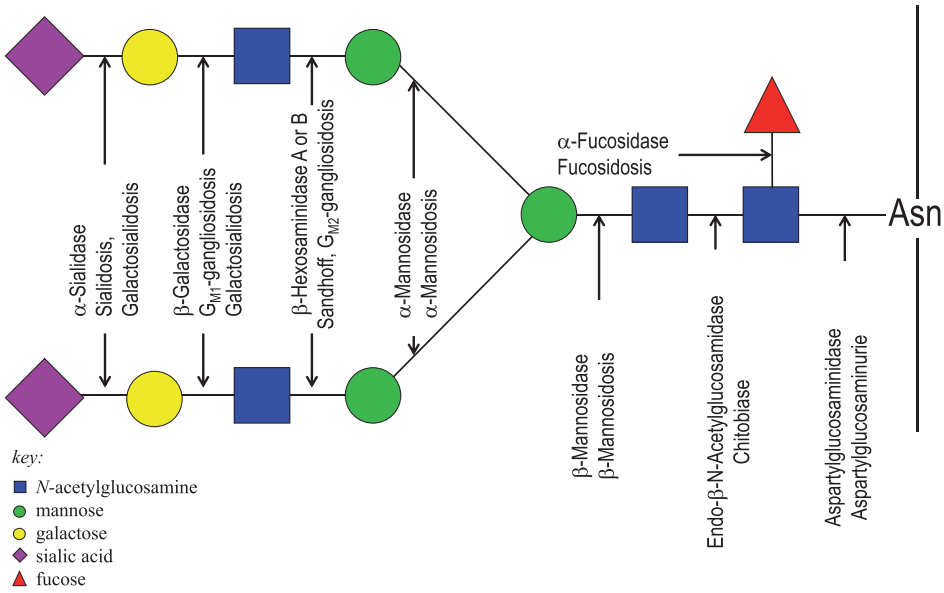

Figure 1-9. Catabolism of complex N-linked oligosaccharides by lysosomal enzymes.

oligosaccharide chain is then sequentially degraded by sialidases and / or α-galactosidases followed by β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase. The remaining Manβ1→4GlcNAc is split by β-mannosidase to mannose and GlcNAc. The resultant monosaccharides are removed from the lysosome by diffusion or with the help of transporter proteins. The highly ordered catabolic pathway explains in part the oligosaccharides found in the diverse Lysosomal storage disorders (LSD).

1.3.2 Catabolism of Ser/Thr-linked glycans in the human lysosome

There are several types of glycans in humans linked to serine or threonine of human glycoproteins. Glycoaminoglycans (GAGs) are attached via a xylosyl-serine linkage. Short mucin-type oligosaccharides are linked via *N*-acetylgalactosamine to serine or threonine. Other glycans are linked via mannose, *N*-acetylglucosamine and fucose to serine or threonine [10]. It is supposed that the catabolism of all these types of *O*-glycosylated proteins happens in the lysosomes. O-glycosylated peptides in urine of patients suffering from Schindler's disease have been characterized by Fourier transform ion cyclotron resonance MS [54]. Little has been published about the enzymology of the lysosomal catabolism of *O*-linked glycans with the exception of glycosaminoglycans of proteoglycans [55]. Most plausible is that the same lysosomal enzymes catalyze the hydrolysis of the same glycosidic linkages in *O*-linked glycans as in other glycoconjugates. A different type of O-linked glycosylation involves the attachment of a single *N*-acetylglucosamine to serine/threonine in nuclear and cytoskeletal proteins is transient and plays a role in intracellular signaling [56]. Its removal is catalyzed by a specific cytosolic *N*-acetylglucosaminidase [57].

1.3.3 Catabolism of glycosphingolipids in the human lysosome

Glycosphingolipids are degraded stepwise from the nonreducing end by exoglycosidases while they are still bound to the ceramide moiety. Since glycosphingolipids have sugar sequences which are in part similar to those found in N- and O-glycans, many of the glycosidases are shared. However, specialized hydrolases and activator proteins are needed for cleaving the glucose-ceramide and galactose-ceramide bonds and other linkages near the membrane.

When a glycosphingolipid contains less than four carbohydrate residues additional noncatalytic sphingolipid activator proteins (SAPs, also called saposins) are essential in human cells for the further degradation by lysosomal hydrolases [58]. In Figure 1-10 the degradation of G_{M1}-ganglioside is schematically represented. The first catabolic step is the cleavage of galactose by the combined action of β-galactosidase and saposin B resulting in leaving G_{M2}-ganglioside. The next hydrolysis step is a combined action of β-hexosaminidase A and the G_{M2} activator protein and this produces G_{M3} ganglioside. The following hydrolysis step is done in a concerted action of sialidase and saposin B and results in lactosylceramide. The concerted action of β-galactosidase and saposins B and C produces glucosylceramide. The last step in degrading the glycan moiety is the combined action of glucosylceramide-β-glucosidase and saponin C to hydrolyse glucosylceramide into glucose and ceramide. Based on this knowledge it has been concluded that free glycan moieties originating from glycosphingolipids are not expected in body fluids like urine.

1.3.4 Lysosomal storage disorder

When one or more of the lysosomal hydrolases are deficient, caused by one or more genetic defects, accumulation of undegraded substrates will happen in the lysosomes. Consequently,

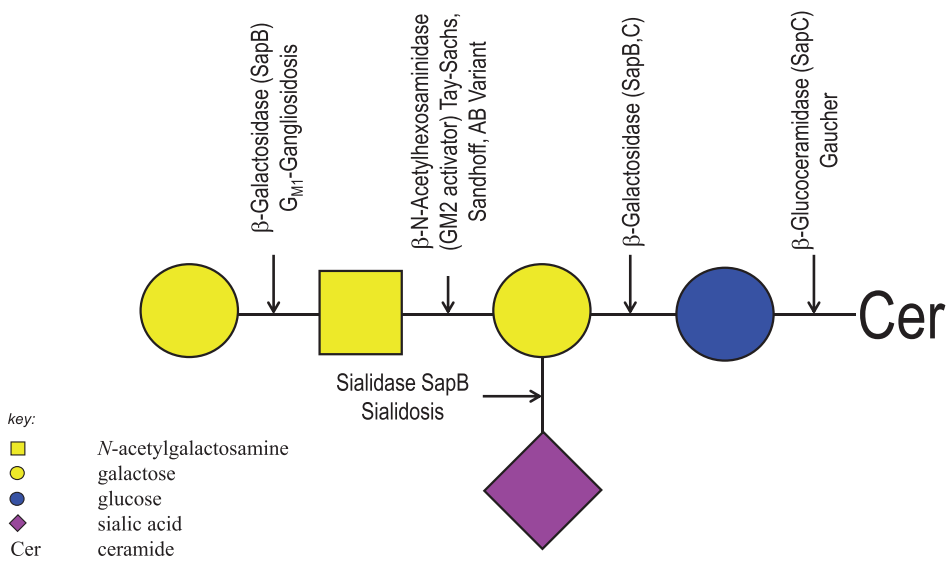

Figure 1-10. Catabolism of the glycan part of glycolipid G_{M1} ganglioside by lysosomal enzymes and cofactors.

lysosomes can swell up to 50% of the cell volume [59] and this will result into damage of many cells. The undegraded substrate will then come free into the extracellular space. Examples of possible stored products are glycosaminoglycans, oligosaccharides, glycopeptides, glycolipids, cholesterols, and sphingomyelins. There have been at least 45 of these deficiencies described forming a group of autosomal recessive inherited lysosomal storage disorders [59,60]. The majority of the lysosomal hydrolases are exoenzymes, so the accumulated and excreted substrates often share the same terminal residue, (see Table 1-5). An extensive review about the lysosome and its disorders has been written by Vellodi [50].

1.3.5 Fucosidosis

Complex type or hybrid type N-glycans may carry fucoses (Figure 1-8). Furthermore fucose can be linked α1-6 to the core *N*-acetylglucosamine that is attached to asparagine. Fucose can be α1-3 or α1-4 linked to *N*-acetylglucosamine in the branches or α1-2 to galactose residues of glycans. The required step in lysosomal degradation of these fucosylated oligosaccharides is hydrolysis of fucose by α-L-fucosidase (EC 3.2.1.51) [62]. A defect of this hydrolase is the cause of fucosidosis. The disorder was initially described by Durand *et al*. in 1968 [63], followed by the report of the defect in α-L-fucosidase by Van Hoof and Hers [64]. Fucosidosis is divided in two distinct phenotypes, but the reality is often more a continuum of severities [65]. The most severe infantile form is type I with an onset of psychomotoric retardation at the age of 3 to 18 months, coarse facies, growth retardation, dysostosis multiplex, neurologic deterioration, and remarkably increased amount of sodium chloride in the patients' sweat. In the milder phenotype, type II, the onset of psychomotoric retardation will be manifest between 1 and 2 years of age. The coarse facies, growth retardation, dysostosis multiplex, and neurologic symptoms are similar or slightly milder than those in type I. The major features that distinguish this milder phenotype from type I are the presence of angiokeratoma, longer survival, and more normal sodium chloride values in sweat.

Fucosidosis is autosomal recessively inherited and the gene symbol for α-L-fucosidase is FUCA1 that codes for the common subunit shared by the multiple forms of α-fucosidase and is situated on chromosome 1p36.11 (MIM 230000). Fucosidosis may be caused by at least 23 different mutations and 4 of these result in an amino acid substitution. For the remaining mutations it is presumed that the result will be unstable or defective mRNA, e.g. premature stop codons, frame shifts, defective splicing, and large deletions [66].

Presumed responsible mutations include the following changes: 188C → T (S63L), 758delA (frame shift), 1201G → T (G401X), 1030ins66 (in-frame insertion), IVS5 + 1G → A (splicing), 229C → T (Q77X), 1145G → A (W382X), 633C → A (Y211X), 421del C (frame shift), 794del C (frame shift), 646del A (frame shift), 340del 10 (frame shift), 549G → A (W183X), 14C → G (P5R), 1988insT (frame shift), 985A → T (N329Y), 1123G → T (E375X), 179G → A (G60D), 451delAA (frame shift), and del exons 7&8 (deletion of 2 exons), 1264C → T (Q422X). All genotypes are homozygote except 229C → T, 1145G → A, 646del A, 451delAA, and 1264C → T. This is evidence for the very high rate of consanguinity found in fucosidosis families [67].

The enzyme defect results in the accumulation and excretion of a variety of fucosylated glycoasparagines and oligosaccharides with core α1–6 linked fucose and / or antenna fucose (Figure 1-11) [68-72].

Table 1-5. Lysosomal disorders. This table is modified from [61] and reprinted with permission.

Disorder	Storage product	Protein defect
Mucopolysaccharidoses (MPS)		
MPS I (Hurler/Scheie)	Oligosaccharides of heparan sulfate, dermatan sulfate	α-Iduronidase
MPS II (Hunter syndrome)	Oligosaccharides of heparan sulfate, dermatan sulfate	Iduronate-2-sulfatase, GlcNAc-P-Transferase
MPS IIIA (Sanfilippo, Pseudo-Hurler polydystrophy A)	Heparan sulfate	Heparan *N*-sulfatase (sulfamidase) GlcNAc-P-Transferase
MPS IIIB (Sanfilippo)	Heparan sulfate	*N*-Acetyl-α-glucosaminidase
MPS IIIC (Sanfilippo, Pseudo-Hurler polydystrophy C)	Heparan sulfate	Acetyl-CoA: α-glucosamide heparan sulfate, *N*-acetyltransferase, GlcNAc-P-transferase
MPS IIID (Sanfilippo)	Heparan sulfate	*N*-Acetylglucosamine-6-sulfatase
MPS IVA (Morquio A syndrome)	Keratan sulfate, chondrotoin-6-sulfate	*N*-Acetylgalactosamine-6-sulfate sulfatase
MPS IVB (Morquio B syndrome)	Keratan sulfate	β-Galactosidase
MPS VI (Maroteaux–Lamy)	Dermantan sulfate	*N*-Acetylgalactosamine-4-sulfatase, (arylsulphatase B)
MPS VII (Sly)	Heparan sulfate, dermatan sulfate, chondroitin-4- and -6-sulfates	β-Glucuronidase
Glycoproteinoses		
Aspartylglucosaminuria	N-linked GlcNAc peptides	*N*-Aspartylglucosaminidase
Fucosidosis	Fucosyloligosaccharides, glycolipides	α-Fucosidase
α-Mannosidosis	Oligosaccharides with α-Mannose at the non-reducing end	α-Mannosidase
β-Mannosidosis	β-Mannosyl-GlcNAc	β-Mannosidase
Sialidosis (Mucolipidosis I)	Sialyloligosaccharides	Exo-α-sialylidase
Schindler disease	Peptides modified with O-linked GalNAc often carrying Gal and Neu5Ac	α-*N*-Acetylgalactosaminidase
Sphingolipidoses		
Fabry's disease	Globotrihexosylceramide (gal-gal-glc-cer), bloodgroup-B substances, digalactosylceramide	α-Galactosidase A
Farber's disease	Ceramide	Ceramidase
Gaucher's disease	Glucosylceramide	β-Glucosidase, saposin-C activator
G_{M1} gangliosidosis	G_{M1} gangliosidoside, galactosyloligosaccharides	β-Galactosidase
Tay-Sachs disease	G_{M2} gangliosidoside and related glycolipids	ß-Hexosaminidase A
Sandhoff's disease	G_{M2} gangliosidoside, G_{A2}, globoside, *N*-acetylglucosaminosyloligosaccharides	ß-Hexosaminidase A and B
Krabbe's disease	Galactosylceramide	Galactosylceramidase
Metachromatic leucodystrophy	Sulfatide, sulfated glycolipids	Arylsulfatase A, Saposin-B activator
Niemann-Pick disease, types A and B	Sphingomyeline	Sphingomyelinase
Other lipidoses		
Niemann-Pick disease type C	Cholesterol and sphingolipids	NPC1 and 2
Wolman's disease	Cholesterol esters and triglycerides	Acid lipase

Table 1-5. continued**.**

Disorder	Storage product	Protein defect
Neuronal ceroid lipofuscinosis		
Glycogen storage disease		
Glycogen storage disease type II (Pompe's disease)	Glycogen	α-Glucosidase
Multiple enzyme deficiency		
Multiple sulfatase deficiency	Glycosaminoglycans, sulfatides	Several sulfatases, Cα-formylglycine-generating enzyme
Galactosialidosis	Gangliosides, sialyloligosaccharides	Cathepsin A secondary deficient β-galactosidase and exo-α-sialidase
Mucolipidosis II/III (I-cell disease and pseudo-Hurler polydystrophy)	Oligosaccharides, mucopolysaccharides, and lipids	UDP-*N*-acetylglucosamine: lysosomal enzyme GlcNAc-P-transferase; secundary deficiency of several lysosomal enzymes
Mucolipidosis IV	Lipids and acid mucopolysaccharides	Mucolipin-1
Lysosomal transport defects		
Cystinosis	Cystine	Cystinosin
Sialic acid storage disease and Salla disease	Sialic acid	Lysosomal transport defect of free sialic acid, Sialin
Other disorders due to defects in lysosomal proteins		
Danon disease	Cytoplasmic debris and glycogen	LAMP2
Hyaluronidase deficiency	Hyaluronan	Hyaluronidase

1.3.6 α-Mannosidosis

The lysosomal storage disorder α−mannosidosis was first reported by Öckerman & Lund [73] and is caused by a deficient lysosomal α-D-mannosidase (EC 3.2.1.24). There are two types of which the most severe phenotype is the infantile type I that shows progressive rapid mental retardation, hepatosplenomegaly, severe dysostosis multiplex, and is mostly mortal between 3 and 12 years of age. The milder juvenile-adult type II form is characterized by a less progressive and milder course with survival into adulthood. The type II is accounting for 10 – 15 percent of cases. This distinction into two types is in fact a continuum of clinical findings [66,74]. α-Mannosidosis is autosomal recessively inherited and the gene coding for the precursors of α-D-mannosidase maps to the human chromosome 19p13-q12. The reported mutation for α−mannosidosis is nucleotide 212A→T which changes amino acid histidine 71 into leucine [75].

The catabolic pathway of high mannose and hybrid type N-linked glycans has been extensively reviewed by Winchester [10]. The pathways for the hydrolysis of these, both type N-glycans by human lysosomal α-D-mannosidase have been elucidated [76]. For human high mannose type glycans the α1-2 linked mannose in the middle branch of $Man_9GlcNAc$, see Figure 1-12, is rather resistant to lysosomal α-D-mannosidase. Hybrid type glycans do not contain this α1-2 linked mannose at that position. The core α1-6 linked mannose is likewise rather resistant to lysosomal α-D-mannosidase. The core tetraose $Man_3GlcNAc$ is no substrate for lysosomal α-D-mannosidase. There is evidence for the existence of a specialized lysosomal α1-6 mannosidase, still active in α−mannosidosis patients [38,77,78]. All these findings do explain the found storage products in urine from α−mannosidosis patients of which the three major mannose rich urinary metabolites are:

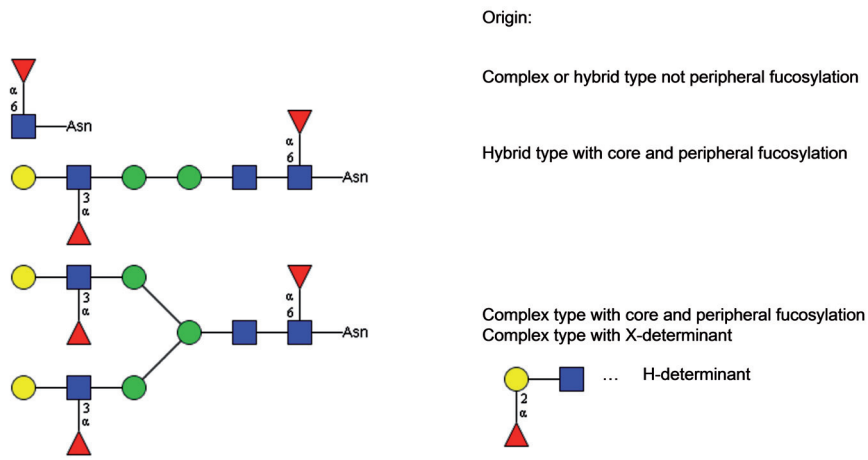

Figure 1-11. Some storage products in fucosidosis. The figure is modified from [10] and taken with permission.

Man(α1-3)Man(β1-4)GlcNAc Man(α1-2)Man(α1-3)Man(β1-4)GlcNAc Man(α1-2)Man(α1-2)Man(α1-3)Man(β1-4)GlcNAc Strecker *et al*. [79] and Yamashita *et al*. [80] have characterized additional mannose-rich oligosaccharides from urine. Until now seventeen mannose-rich oligosaccharides have been characterized from pooled urines of two mannosidosis patients [81,82], of which sixteen had been reported earlier [80,83]. All these mannose rich oligosaccharides, found in urine samples from α-mannosidosis patients, are endo-*N*-acetyl-β-glucosaminidase cleaved products. The major metabolite Man(α1-3)Man(α1-4)GlcNAc in urine is most probably derived from incompletely digested complex type glycans. The presence of a lysosomal (α1-6)mannosidase, still active in affected patients, supports this assumption [10,76].

1.3.7 Sialidosis and Galactosialidosis

Many glycan structures in glycoconjugates contain *N*-acetylneuraminic acid (Neu5Ac), one of the sialic acids, with a linkage of α2–3 or α2–6. The cause of sialidosis is a deficient acid hydrolase exo-α-sialidase (EC 3.2.1.18) [84]. Exo-α-sialidase (sialidase) cleaves both type of linkages α2–3 and α2–6 [85].

In galactosialidosis both sialidase and β-galactosidase (EC 3.2.1.23) are deficient. The deficiency of sialidase and β-galactosidase is a secondary effect of a defect of another enzyme, an associate in the large multi-enzyme complex protective protein/carboxypeptidase C (PPCA), cathepsin A.

1.3.7.1 Protective protein/carboxypeptidase C (PPCA)

The mammalian multi-enzyme complex protein/carboxypeptidase C has both protective and catalytic functions. PPCA is an association of sialidase, β-galactosidase, carboxypeptidase C (EC 3.4.16.5), and recently indications have been found that *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) (EC 3.1.6.4) also belongs to the multi-enzyme complex [86,87].

PPCA protects sialidase and β-galactosidase from fast proteolysis in the aggressive environment of the lysosome, and the interaction of sialidase with PPCA is needed for optimal activity of sialidase [87-93] (see table 1-6).

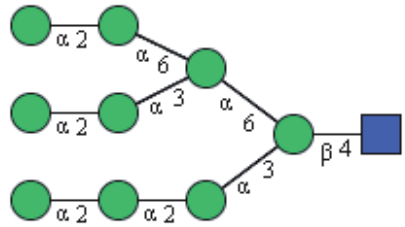

Figure 1-12. High mannose $Man_9GlcNAc$ cleavage product generated from glycoproteins by endo-*N*-acetyl-β-glucosaminidase.

1.3.7.2 Sialidosis

Due to the considerable heterogeneity of the clinical phenotypes observed a proper delineation of sialidosis is difficult. Nowadays two subtypes of sialidosis are distinguished [94,95]. The milder type I is characterized by ocular cherry-red spot and development of myoclonus at the age of 20 – 40 years. The onset of type I occurs at the age of 2 – 25 years. In more than 50% of the affected patients seizures, hyperreflexia, and ataxia are observed [96-98]. The rather severe type II sialidosis is distinguished from type I by an early onset, often at the age of 0 – 2 years. The phenotype shows ocular cherry-red spot, mental retardation, myoclonus, visceromegaly, dysotosis multiplex, hydrops/acites, and skeletal dysplasia [84,99,100]. Sialidosis is autosomal recessively inherited and the gene at location 6p21 is involved. The presumed responsible mutations are 1258G→T, amino acid change H337X; 401T→G, amino acid change L91R; 1337delC, frame shift; 7insACTC, frame shift; 779T→A, amino acid change F260Y; 1088T→C, amino acid change L303P [101,102]. As an effect of deficient sialidase in both subtypes excessive amounts of sialyloligosaccharides in urine of up to 800 x normal levels are observed [103]. Most compounds are endo-β-*N*-acetylglucosaminidase-cleaved products of complex-type sialylated *N*-glycans [104-106]. In 70% of the cases the Neu5Ac is α2–6 linked to galactose and α2–3 linked in 30% [107].

1.3.7.3 Galactosialidosis

For galactosialidosis three phenotypes are distinguished. Firstly, the severe early infantile type that shows onset between birth and 3 months of age with fetal hydrops, neonatal edema, kidney involvement, coarse facies, inguinal hernias, and telangiectasias, small dilated blood vessels near the surface of the skin. This phenotype generally leads to death before the age of 24 months. Telangiectasias has seldom been observed in the other two phenotypes.

Secondly, a mild type is observed named the late infantile type. The symptoms initiate in the first months after birth. Observations at onset are coarse facies, hepatosplenomegaly, and dysostosis multiplex, especially affecting the spine. Often cherry-red spots and / or corneal clouding are observed. The third phenotype is juvenile/adult type. This type shows a variable, broad continuous spectrum of severity of the course and of the age of onset. First symptoms become visible at 1 to 40 years of age with an average of 16 years. Often coarse facies are found, spinal changes are observed whilst dysostosis multiplex seems rare. The most observed neurologic abnormalities are myoclonus, ataxia, seizure, and progressive mental retardation. The clinical phenotype of galactosialidosis is very similar to that of sialidosis most probably due to the deficiency of sialidase which they have in common [108].

Galactosialidosis is autosomal recessively inherited and is genetically unrelated to sialidosis as the primary defect is in PPCA (Table 1-6).

The gene that transmits the disorder is localized on chromosome 20q13.1 [110]. Mainly single-base substitutions or splice-junction defects have been identified as cause.

The major clinical phenotype is caused by the deficient activity of sialidase. The resulting urinary excretion of sialyloligosaccharides has been reported to be similar to that found in sialidosis [111,112]. Comparative studies of oligosaccharides excreted by galactosialidosis and sialidosis patients have yielded conflicting results: Takahashi *et al*.[107] found excessive galactosyl-terminated oligosaccharides in galactosialidosis patients, while these compounds were not detected by van Pelt *et al*. [112,113].

1.3.8 G_{M1}-gangliosidosis

The lysosomal storage disorder G_{M1}-gangliosidosis was first described by Suzuki *et al*. [114]. G_{M1}-gangliosidosis is divided into three phenotypes. Type 1 or infantile type shows an onset mostly before 6 months of age. The development of the infant shows delay in the first 6 months and after this period severe brain damage becomes obvious. Macular cherry red spots, hepatosplenomegaly, dysmorphism, and generalized skeletal dysplasia are observed. Within two years after onset the disease is fatal. Type 2 or late infantile/juvenile type G_{M1}-gangliosidosis shows an onset between 7 months and 3 years of age and presents with a heterogeneous phenotypic appearance. The range of symptoms is the same as for type 1 but not all of them are observed in affected patients. The type 2 of the disorder is fatal within 5 years of age. The type 3 or adult/chronic form shows an onset from 3 to 10 years of age. Macular cherry red spots, dysmorphism, and hepatosplenomegaly are absent. Generalized skeletal dysplasia is observed. Type 3 is fatal after approximately 30 years of age. The 3 types of G_{M1}-gangliosidosis have the storage of ganglioside G_{M1} and oligosaccharides derived from glycoproteins in common [115,116]. G_{M1}-gangliosidosis is caused by the deficiency of β-galactosidase (EC 3.2.1.23) and is autosomal recessively inherited. The gene of human β-galactosidase is localized on chromosome 3p21.33. Heterogeneous gene mutations have been found. Five common mutations have been described: for type 1 R208C in American patients [117] and R482H in Italian patients [118]; for type 2, R201C in Japanese patients [119,120]; and for type 3, I51T in Japanese patients [119]. Galactosyl oligosaccharides are found in urine samples of patients suffering from G_{M1}-gangliosidosis [121-124]. A correlation has been established between severity of the disease and the concentration of the excreted galactosyl oligosaccharides [123,124].

Table 1-6. Relation of PPCA to LSDs [109].

Enzyme or protein	deficiency leads to	Disorder
Sialidase	⟶	Sialidosis
↑ activation		
PPCA	⟶	Galactosialidosis
↓ stabilization		
β-Galactosidase	⟶	G_{M1}-gangliosidosis

1.3.9 G_{M2}-gangliosidosis

There are three essential lysosomal polypeptides needed for the cleavage of the *N*-acetylgalactosamine at the non-reducing end for metabolic recycling of different glycan moieties such as G_{M2}-ganglioside. The G_{M2}-ganglioside requires to be complexed with the substrate-specific liftase G_{M2}-activator protein [125] to facilitate enzymatic hydrolysis of the GalNAc at the non-reducing end by β-hexosaminidase (EC 3.2.1.52). There are two isoenzymes of β-hexosaminidase: β-hexosaminidase A (Hex A) with heterodimeric structure αβ, and β-hexosaminidase B (Hex B) with homodimeric structure ββ. Hex A is the only isozyme that can hydrolyze G_{M2}-ganglioside *in vivo* [126]. The G_{M2}-ganglioside/G_{M2} activator protein complex only interacts with Hex A. Consequently there are three different variants of G_{M2}-gangliosidosis and they are caused by defects in different genes. Sandhoff *et al*. [127] proposed a classification based on the β-hexosaminidase isoenzyme that is still active in tissue of affected patients. In Tay-Sachs disease and its variants (variant B) Hex A activity is deficient and the deficiency is caused by mutations of the HEX A gene encoding for the α subunit. Sandhoff disease and its variants (variant 0) have a combined deficient activity of Hex A and Hex B and are caused by defects in the HEX B gene encoding for the β subunit common in Hex A and Hex B. In the AB variant the G_{M2}-activator protein is defect which is caused by mutations in the GM2A gene. Hydrolysis of ganglioside G_{M2} by Hex A is impaired by the absence or defective formation of the G_{M2}-activator protein/ganglioside G_{M2} complex [128].

The above-mentioned way of describing G_{M2}-gangliosidosis is impractical for describing the large spectrum of clinical variance observed. Classification by clinical designations recognizes the dominance of the encephalopathy rather than the age of onset as the primary clinical delineator [129]. The clinical phenotype of infantile acute G_{M2}-gangliosidosis is indistinguishable from Tay-Sachs disease, Sandhoff disease, and G_{M2}-activator protein deficiency (AB variant). The first signs of onset are often only recognized in retrospect but start at 3 to 5 months with mild motor weakness. Clinical observations are regression and even loss of acquired mental skills, organomegaly, ophthalmology, macular cherry red spot, macrocephaly, and death between 1 to 2 years of age.

The next phenotype is the late-onset form of G_{M2}-gangliosidosis. This phenotype is not known for variant AB. The clinical phenotype varies widely in the late onset type of Tay-Sachs and Sandhoff disease. Onset varies from late infantile period to adult age. Macular cherry red spot are less frequent observed. In some patients mental capacity can be well preserved, although often masked by dysarthria. The onset of subacute G_{M2}-gangliosidosis starts between 2 and 10 years of age by the development of ataxia and incoordination. Clinical phenotype includes regression in speech and life skills, dementia, psychomotor deterioration, increasing spasticity, and loss of vision. A vegetative state with decerebrate rigidity and involuntary extension of the upper extremities in response to external stimuli indicating brain stem damage, develops around 10 to 15 years of age, followed within a few years by death, usually due to intercurrent infections. Patients suffering chronic G_{M2}-gangliosidosis show onset from childhood to adulthood. G_{M2}-gangliosidosis is an autosomal recessively inherited disorder. HEXA maps to chromosome 15, while HEXB and GM2A maps to chromosome 5. Infantile, subacute, or chronic disease forms of G_{M2}-gangliosidosis can be distinguished in mutations of DNA. The estimated heterozygote frequencies in the general population are 0.006 for HEXA and 0.0036 for HEXB mutations. In some ethnic groups, the observed carrier frequencies are remarkably higher. The heterozygote frequency for HEXA mutations is 0.033 for the Ashkenazi

Jewish population [129,130]. For all variants of G_{M2}-gangliosidosis the major neural storage compound is ganglioside G_{M2} [130-132]. The ubiquitous storage compounds in Sandhoff disease are oligosaccharides derived from glycoproteins. Blockage of the N-glycan catabolism results in accumulation of β-endo-*N*-acetylglucosaminidase cleaved oligosaccharides carrying a single *N*-acetylglucosamine residue at the non reducing end which are found in the urine of Sandhoff disease patients [129,133-136].

1.3.10 Diagnostic methods in lysosomal storage disorders

The group of lysosomal storage disorders contains a subgroup of disorders called heteroglycanoses which are characterized by the excretion of glycans and / or glycoconjugates in urine. The only accepted method to determine these disorders unambiguously is the determination of the specific (reduced) enzyme activity. Not all clinical laboratories are equipped for these expensive and difficult assays [137]. Another way is to screen for excess urinary glycans with paper chromatography [138] or thin layer chromatography [139]. These methods are often unreliable [140,141]. Interpretation of a TLC pattern of excreted oligosaccharides is sophisticated and needs extraordinary skills [137]. HPLC appears to be an attractive alternative that could overcome some of the limitations of TLC pattern interpretation. Derivatization of glycans is necessary for UV or fluorescence detection or to obtain interaction with stationary phases commonly used in HPLC [142-144]. Derivatization of saccharides is depending on reducing ends for reductive amination reactions [145,146]. HPAEC and PAD do not depend on derivatization and are therefore more broadly applicable for characterization of saccharides [14,147,148]. Hommes *et al*. have reported on the diagnostic use of HPAEC-PAD for heteroglycanoses disorders [149].

1.4 SCOPE OF THE THESIS

High performance anion-exchange chromatography with pulsed amperometric detection is a well-accepted method for separating and characterizing carbohydrates [13,14]. Downscaling of column dimension is a viable approach in glycan analysis technology to achieve better sensitivity for the analysis of samples available in limited amount. The miniaturization of the column comes together with lower eluent flow rates, which is advantageous for mass spectroscopy. HPAEC hyphenated to an ion trap mass spectrometer is a strong analytical combination for identifying carbohydrates and for performing partial structural elucidation. This thesis reports on the development of a capillary scale HPAEC using prototype capillary columns as well as a prototype desalter which allows the hyphenation to ESI-MS. To investigate the applicability and value of capillary HPAEC in biomedical research, urine and ascitic fluid samples from patients suffering from a range of disorders that affect the lysosomal carbohydrate catabolism were investigated. In addition, amniotic fluid samples from mothers carrying a diseased child were analyzed. The selectivity of HPAEC in combination with the better sensitivity of the new platform and the informative data generated by MS detection has provided new insights into the catabolism of glycoconjugates. Thus, this thesis demonstrates the usefulness of HPAEC-PAD-MS as an analytical tool in oligosaccharide analysis, both with regard to glycan structural elucidation and to glycan profiling.

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