6

GENERAL DISCUSSION

6.1 LIQUID CHROMATOGRAPHY HYPHENATED TO MASS SPECTROMETRIC DETECTION

Glycoconjugates have different biological functions which often depend on the structure of the glycan part. Hence, for fully determining structure-function relationships the elucidation of the glycan structure is essential. Isomeric carbohydrates often have different biological functions too, and it is therefore critical to be able to distinguish between these forms, for example by separating them chromatographically prior to performing mass spectrometric analysis. Liquid chromatography is the technique most commonly used for separating carbohydrates, the major separation principles being reversed-phase chromatography (RPLC), porous-graphitized-carbon chromatography (PGC), hydrophilic interaction liquid chromatography (HILIC), ion-exchange chromatography (IEC), and high-performance anion-exchange chromatography (HPAEC). Of these methods, RPLC, PGC and HILIC are well suitable for MS coupling, while MS coupling of IEC and HPAEC has so far not been straightforward. This thesis reports on major advances in HPAEC-PAD-MS with the successful development and application of the first capillary scale system (column 400 µm I.D.) with online desalting and online ESI-MS coupling suitable for high-sensitivity analysis of glycans. Together with the well-established high separation power and selectivity of HPAEC, this has resulted in an attractive glycoanalytical tool which may be broadly applied in the future.

Several components of the system had to be adapted to fulfill the requirements of capillary HPAEC-PAD. The original amperometric detection cell was modified to minimize its void volume. The band dispersion, caused by the modified amperometric cell, was investigated by separating an inulin solution with an acetate gradient, and comparing its chromatographic resolution with that obtained by a standard HPAEC-PAD equipped with a narrow-bore column (2 mm I.D.). The obtained chromatograms from these two systems showed very similar band dispersion, indicating that the minimization of the void volume made the electrochemical detection cell suitable for capillary-scale use (Chapters 2 and 3). While with the narrow-bore system (chapter 2) a minimum detection limit (MDL) of 120 fmol was obtained, the capillary-scale setup resulted in an MDL of 22 fmol (Chapter 3), indicating that the capillary-scale setup resulted in increased sensitivity when employing PAD detection. Finally, the modified amperometric cell proved to be appropriate for the sensitive detection of native carbohydrates in capillary-scale chromatography.

A bigger challenge than the PAD analysis was the development of a capillary desalter to make hyphenation of capillary HPAEC to MS possible. To enable an online setup, the desalter had to convert the eluting solution into an MS-compatible solvent. The challenge was to obtain enough desalting efficacy to remove from the solution the sodium ions – arising from sodium hydroxide and sodium acetate present in the applied eluents - without sacrificing too much of the obtained chromatographic resolution. The desalting capacity obtained was 225 mM Na^+ at 10 µl/min flow rate (Chapter 3). This capacity is high enough to desalt eluents used for the elution of glycans with a maximum of three negative charges, e.g. three sialic acid groups contained in oligosaccharide structures (Chapter 4). The change in resolution caused by the developed desalter was determined by comparing the results of a gradient separation of an inulin solution with the capillary amperometric detection cell directly following the capillary column, with that of the same separation with the desalter installed between the column and the amperometric cell. This comparison demonstrated that the capillary-scale online desalter

only resulted in a minor loss of chromatographic resolution and was, therefore, suitable for MS coupling of capillary-scale HPAEC in oligosaccharide analysis (Chapter 3).

By downscaling the column dimension to 400 µm I.D. an important sensitivity gain was obtained in electrospray ionization and MS detection. This improved sensitivity can be essential for biomedical research. The minimum detection limit (MDL) obtained for underivatized glycans with capillary-scale HPAEC-MS was found to be 160 fmol using a conventional electrospray ionization source (Chapter 3), while nano-scale HILIC-MS approximately with an online-nano electrospray ionization source gives an MDL of 5 fmol [1]. This difference in MDL largely reflects the difference in column diameters (400 µm I.D. for HPAEC, 75 µm I.D. for HILIC) indicating that the sensitivity of HPAEC-MS and HILIC-MS might be similar if both techniques are performed with identical column dimensions, and identical mass spectrometric detectors.

The high pH of the eluent used in HPAEC causes a fast conversion of anomers, which suppresses anomer separation completely, resulting in an excellent peak performance [2]. PGC often separates anomeric forms [3], which phenomenon can interfere with the necessary resolution between different carbohydrates. This effect can often be reduced by elevating the column temperature. Likewise, HILIC separations of reducing-end oligosaccharides performed at acidic pH show at least partial anomer separation, thereby complicating the analysis of complex samples [1].

The analysis of urinary oligosaccharides by HPAEC-MS as performed in this thesis required only a single sample pretreatment step, namely solid-phase extraction with porous graphitized carbon material. Eluates were dried and reconstituted in small volumes of water, allowing the injection of analytes in high concentrations, as a result of which we obtained an in-depth analysis of complex samples. In contrast, HILIC separations of oligosaccharides are performed with very high concentrations (80% and higher) of organic solvents as starting condition [4], which reduces the solubility of oligosaccharides and may cause problems in the event of high concentrations of glycans, especially at low temperatures (such as may occur in the cooled tray of an autosampler). During the phase of method development, our group has indeed experienced irreproducibility of results due to the initial high organic solvent concentration.

HPAEC is known for its ability to separate glycan isomers differing in linkage type and compositional order [2,5-7], and the capillary HPAEC-PAD-MS indeed gave a complete separation of the 6-arm and the 3-arm monoantennary complex oligosaccharides both with galactose and with *N*-acetylglucosamine at the non-reducing end (Chapters 3 and 5). Many other examples of the separation of structural isomers of complex *N*-glycans are reported in Chapter 4. This chapter reports mainly about the separation of sialylated glycans. The observed separation order of oligosaccharides with α2-3- or α2-6 linked sialic acid was in accordance with that described in literature [8]. In addition, we observed charge-based separation of oligosaccharides with a terminal, reducing-end hexose and of related species with a terminal aldohexonic acid instead (Chapter 4): the additional charge caused by the aldohexonic acid (generated by C_1-oxidation of hexose) consistently resulted in an increase of retention time, which is primarily based on ionic interactions. At least two features are responsible for the selectivity of glycans in HPAEC: (i) the difference in acidity of the various hydroxyl groups [9] and (ii) the accessibility of oxyanions of the oligosaccharides to the functional groups of the stationary phase [5]. PGC and HILIC also show separation of glycan isomers, but this isomer separation has been determined to be based on other interaction mechanisms than that of HPAEC. In general, PGC shows selectivity on the basis of glycan size, charge of acidic glycans,

and the 3D-structure of glycans resulting from linkage between the sugar monomers [10-12]. A glycan with a relatively planar structure tends to have a longer retention with PGC material than glycans with a more globular structure, which is due to differences in contact area between analyte and graphite surfaces [10,13]. The selectivity of HILIC for glycans is mainly based on hydrophilic interaction between the bulk eluent and a water-rich layer, partially immobilized on the stationary phase [14,15]. The observed selectivity rules for glycans in HILIC show separation on glycan size, and linkage between the sugar monomers [16,17]. So, the isomer separation of HPAEC is different from that of PGC and HILIC, and therefore these three kinds of chromatography selectivities are orthogonal to each other.

The research described in this thesis was done on a prototype capillary ion chromatograph. Our challenge in the coming years will be to make capillary-scale HPAEC-MS more generally available. In 2010 the first commercially available capillary ion chromatograph was introduced [18], but this system did not yet provide all the modules necessary for HPAEC-MS of oligosaccharides, such as a capillary-scale ternary gradient pump and the required capillary-scale HPAEC separation columns. The main field of application for the present instrument is the analysis of small anions and cations and this field can be of potential value for the analysis of smaller ionic compounds such as nucleotides, sugar phosphates, and organic acids in the fields of metabolomics and metabonomics [19]. The instrument includes a capillary desalter, a capillary amperometric detection cell, a capillary eluent generator, and an isocratic capillary pump, and this combination of modules is suitable for analyzing mono- and disaccharides with capillary HPAEC-PAD-MS [20,21], but not for larger, potentially charged oligosaccharides.

Instead of manually checking the desalting efficacy prior to the start of a sample analysis sequence only once, a more robust approach would be an online desalting monitor that protects the mass spectrometer from possible contamination caused by insufficient desalting. Such a monitor could contain the desalter, a connection for introducing make-up liquid, and an electronic output to stop the chromatograph to prevent salt from entering into the electrospray ionization source, thus contaminating the mass spectrometer.

To obtain a higher sample throughput while retaining selectivity and resolution, a faster separation would be desirable. One possible way to achieve this would be the development of ion exchange monolithic columns [22], which has already started for small ions [23]. Another possibility would be the use of smaller particles than the 4-µm materials which have just been introduced for IC. Particles of the envisaged size (≤ 2-µm) are already in use for HPLC [24] in RP and HILIC columns, resulting in vastly increased peak capacities [25,26]. However, the use of ion exchange columns packed with smaller particles requires an ion chromatography system capable of running at higher pressures than 34.5 MPa, which is the present pressure limit of ion chromatographs.

To develop optimal LC methods for monolithic columns and columns packed with smaller particles, the kinetic-plot method is a valuable tool [27]. A kinetic-plot is a graphical approach allowing the selection of column specifications (i.e. optimum particle size and column length) and LC conditions (operating pressure and temperature) to generate a specific number of plates or peak capacity in the shortest possible analysis time [27]. Kinetic plot measurements for small ions have been published by Causon *et al.* [28,29] for an analytical-scale column. Wouters *et al.* are now carrying out similar measurements for a capillary-scale ion-exchange column [30].

An important part of the instrumental setup for this work obviously was the MS. Again, enhanced sensitivity is vital in cases where only very small sample amounts are available. In

the last decades many developments in mass spectrometers have led to a strongly improved sensitivity of MS instruments by decreasing the noise level and increasing the signal. The expected gain in sensitivity which would be realized by using a state of the art MS instrument instead of the MS used for the research reported in this thesis is approximately two orders of magnitude and should make new high-sensitivity applications accessible. Further enhancement of the sensitivity could be realized by further miniaturization of the column. Biomedical research makes a more and more frequent use of nano-scale liquid chromatographs, and a similar development is expected for ion chromatography. The main challenge here is to minimize the void volume, caused in particular by the desalter. A possible approach would be to integrate the separator column and the desalter, thus avoiding fitting and tubing materials [31]. The improvements in instrumentation setup, as described above, could be of high value for limited sample amounts, such as in studies on posttranslational modification of proteins. An example of such a modification would be provided by glycans released from extracted electrophoretic bands. Another example of applications where sensitivity is of importance is in the analysis of ionic compounds and carbohydrates in smaller objects, such as biopsy samples.

6.2 LYSOSOMAL STORAGE DISORDERS

The capillary-scale HPAEC-PAD-MS system developed in this study was used for the characterization of free glycans in urine, amniotic fluid and ascitic fluid samples. The analyses have highlighted relevant glycan structures which are potential markers for each of the different lysosomal storage disorders examined. While we here applied our newly developed capillary-scale HPAEC-PAD-MS for the characterization of disease-associated urinary glycans, it should be stated that other modern glycoanalytical techniques would most likely be similarly suitable to reveal the characteristic urinary glycan profiles from urine as well as other body fluids. Such alternative techniques include the analysis of fluorescently labeled glycans by e.g. UPLC with fluorescence detection (HILIC, reverse-phase or graphitized carbon), capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) as well as various types of mass spectrometry including MALDI-TOF-MS of native or derivatized glycans, and various LC-MS approaches [11,32]. It should be stated, however, that the distinct properties of these detection methods are certainly expected to influence the (sub-)set of urinary glycans which is amenable to analysis by the various methods. For example, relying on reducing-end labeling via aldehyde groups will preclude the detection of the C_1-oxidized glycans described in this thesis. Moreover, the use of a powerful separation technique (such as in LC-MS, UPLC with fluorescence detection, or CGE-LIF) will favor isomer differentiation [16,25,33].

On another note, when aiming at the glycan analysis from a large set of urinary samples, a two-step analytical approach may be advantageous: analysis of the urinary glycan profiles may be achieved by a fast, high-throughput method such as HPLC with fluorescence detection or CGE-LIF, and only a selected set of samples where the structural assignment cannot be performed satisfactorily using the profiling methods would be subjected to (tandem) mass spectrometry as a more costly and time-consuming in-depth analytical method.

While many of glycan structures which were identified in this thesis had already been reported in the literature for the different disorders investigated, it is remarkable that in the various body

fluids from galactosialidosis patients new glycan structures with a reducing-end hexose were found, which structures are assumed to be derived from glycolipids. The levels of oligosaccharides with reducing-end hexose found in the body fluids were surprisingly high and they are comparable to the levels of glycans derived from glycoproteins. It is unclear why these oligosaccharides have not been observed in earlier studies. A possible explanation can be the strong focus in earlier research on *N*-glycans and *O*-glycans [34] and the use of a different analytical approach than in this thesis [35-37]. The analytical approach used in earlier studies required extensive sample cleanup and was targeted to sialyl-oligosaccharides [35,36,38]. In another study the urine sample to be analyzed was first fractionated by gel permeation chromatography (GPC). Structural eludication of one fraction by NMR analysis was reportedly not successful [37]. Obviously, in these studies some glycan may have been missed due to extensive sample cleanup procedures and the targeted nature of the approaches. In contrast, the approach used in this thesis, only requires desalting of the urine samples with PGC material and no further fractionation.

The observed glycan structures which would appear to be derived from glycolipids could be explained by the possible existence of an endoglycosylceramidase. This endoglycosylceramidase would be involved in an alternative glycosphingolipid catabolic pathway, although such an enzyme has not yet been described for vertebrates, as discussed in Chapter 4 of this thesis. In addition to the glycan structures mentioned, the same samples were also observed to contain the aldohexonic acid forms of these glycolipid-derived glycans. Furthermore, some aldohexonic acid glycans were also detected in the sialidosis urine when the MS was operated in the negative mode.

It should be emphasized that in the studies done for this thesis, only free glycans in body fluids were examined. It would be worthwhile to investigate the possible presence of urinary glycopeptides in various lysosomal storage disorders. Additionally, the analysis of intact glycolipids in the urines would help to obtain a comprehensive picture of storage products. Further investigation of the postulated endoglycosylceramidase would be helpful to gain better understanding of the catabolism of glycolipids in the human body. With the investigation into glycopeptides and the endoglycosylceramidase activity in combination with the enzyme defect, more could be learned about the catabolism in the lysosomes. Another potentially valuable approach would be to examine the oxidation reaction of glycans derived from glycolipids into their aldohexonic acid form to potentially reveal new insights in glycoconjugate degradation pathways.

In conclusion, using the newly developed capillary HPAEC-PAD-MS setup we were able to determine and characterize a number of oligosaccharides that had not previously been reported in various types of body fluids. The increased sensitivity achieved by using the capillary-scale HPAEC-MS also allowed detection of a number of sulfated *N*-glycans. The developed analytical method together with the semi-targeted approach to the MS data analysis were successfully applied to urine and ascitic fluid samples of patients suffering from lysosomal storage diseases and to amniotic fluid samples from the mothers carrying a fetus suffering from an LSD. Finally it can be stated that detection of urinary oligosaccharides may not only represent a suitable method for the diagnosis of lysosomal storage diseases, but that, in addition, monitoring of the profiles and amounts of these glycans during the therapy of LSDs may provide a powerful tool for assessing therapeutic efficacy.

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