Glycoconjugate Journal 16, 45–58 (1999)
© 1999 Kluwer Academic Publishers. Manufactured in The Netherlands

Liquid chromatography "on-flow" ^{1}H nuclear magnetic resonance on native glycosphingolipid mixtures together with gas chromatography/mass spectrometry on the released oligosaccharides for screening and characterisation of carbohydrate-based antigens from pig lungs

Annika E. Bäcker[1]*, Sara Thorbert[1], Olivier Rakotonirainy[2], Eva C. Hallberg[1], Anne Olling[1], Mikael Gustavsson[1], Bo E. Samuelsson[1] and Bassam Soussi[2]

[1]*Institute of Laboratory Medicine, Department of Clinical Chemistry & Transfusion Medicine,* [2]*Bioenergetics Group Wallenberg Laboratory, Sahlgrenska universitetssjukhuset, SE413 45 GÖTEBORG, Sweden.*

Glycosphingolipids were prepared from pig lung and pooled into two fractions with (*i*) ≤3 sugar residues, and (*ii*) ≥3 sugar residues. Oligosaccharides were prepared and used for gas chromatography, gas chromatography/mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. The glycolipid fractions *i* and *ii* were further characterised and purified using a novel method based on high performance liquid chromatography "on-flow" proton nuclear magnetic resonance. The LC "on-flow" NMR technique showed good chromatographic separation and gave NMR spectral information which could be used as guidance for pooling of the separated mixture glycolipids. Conventional ^{1}H NMR, thin layer immunostaining, gas chromatography, gas chromatography/mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry were used to characterise the glycolipids and to validate LC-NMR spectral data.

Keywords: **gas chromatography/mass spectrometry, glycosphingolipids, liquid chromatography-NMR spectroscopy, pig, xenotransplantation.**

Introduction

The hyperacute rejection (HAR) associated with discordant xenotransplantation (e.g. transplantation from pigs to humans) is closely related to carbohydrate antigen epitopes on the cell surfaces. Since the pig antigens are mainly located in the endothelial cells, the endothelium rich pig lung is a useful source for characterising such antigens. Pig lung is also a possible donor organ in future human xenotransplantation. The analysis of xenotransplantation-related carbohydrate antigens [1–3] requires great effort and time in preparation of the corresponding glycolipids (GSL) (see material and methods). The critical amount of sample for characterisation of components in a mixture is sometimes jeopardised because of limited organ tissue available and due to preparational losses of less abundant, more polar glycolipids. Thus, there is a need to develop an improved, standardised and reliable analytical technique to monitor and rationalise GSL purification.

The liquid chromatography-nuclear magnetic resonance (LC-NMR) technique has been used for detection and characterisation of small molecules such as drug metabolites in urine and bile during recent years [4–7]. We have earlier developed the LC-NMR technique for mixture analysis of native glycolipids [8,9] and shown its feasibility in analysing standardised mixtures of well-defined smaller glycosphingolipids [9]. In this study, LC-NMR was used for rapid characterisation screening of individual components in a glycolipid mixture, as well as an advanced monitoring tool for pooling specific fractions.

The LC-NMR spectra can be used for pooling and structural characterisation of glycosphingolipid mixtures. This is a reliable choice compared to the commonly used TLC

*To whom correspondence should be addressed. Tel +46 31 342 2174; Fax: +46 31 41 76 31; e-mail: annika.backer@ss.gu.se

assay. The latter assay is based on glycolipid migration by organic solvents on a high performance thin layer chromatography (HPTLC) plate and visualisation by a chemical reagent. In the case of LC-NMR, the silica column separates GSLs on the basis of sugar chain length, ceramide differences, and the sterical conformation of the sugar chain (as for the TLC plate), but the column also introduces the solvent gradient system as an important separation factor. The spectra, in turn, give structural information about such factors as α- and β-anomeric protons, which indicate binding positions and binding configuration as well as number of monosaccharides in the carbohydrate chain.

Glycosphingolipids are denoted by blood group activity, the epitope name, the number of monosaccharides in the chain and the core saccharide chain type. For example, H-5-2 denotes a five-sugar blood group H structure based on a type 2 chain. The nomenclature used to describe fragmentation and ions is in accordance with Domon and Costello [22].

Materials and method

Preparation of glycosphingolipids

A total non-acidic glycolipid (GSL) fraction was isolated from four pig lungs using the method of Karlsson [10]. The lungs were freeze dried (dry weight 231g) and extracted with organic solvents using chloroform/methanol mixtures in order to release the lipid-containing components. The isolation procedure included alkaline methanolysis, silica columns, DEAE-cellulose columns and acetylation.

HPLC fractionation of glycosphingolipid mixtures

The non-acid glycosphingolipid mixture was fractionated by silicic-acid column chromatography (Polygosil® 60-5, Machery-Nagel, Germany) [11] on a high performance liquid chromatography (HPLC) system (LKB 2150 & 2152, Sweden) using a linear gradient from 80/20/1 to 40/40/12 (by volume) of chloroform/methanol/water [11]. TLC analysis was used for monitoring and controlling column chromatography. A constant flow rate of 2mL/min was used over 280 min. Fractions of 4mL were collected. Fractions containing glycolipids with ≤ 3 sugar residues (non-polar, *i*) in the carbohydrate chain according to thin-layer mobility were pooled together (126.1mg), as were fractions containing glycolipids with ≥ 3 sugars (polar, *ii*) in the carbohydrate chain (129.2mg).

Ceramide glycanase-cleavage of glycosphingolipids

200 µg of each polar glycolipid mixture was mixed with 30 µL (30mg/mL) sodium cholate (Sigma) in chloroform/methanol, 2/1 (by volume). The samples were dried under a nitrogen stream, re-suspended in 200 µL 0.1M sodium acetate buffer (pH 5.0) and 5mU ceramide glycanase (Ceramide glycanase from leeches (EC 3.2.1.4.5) Boehringer Mannheim, Germany) and finally incubated for 48 hours at 37 °C [12]. The reaction mixtures were passed through prewashed C18 reversed phase SepPak cartridges (Waters Associates, USA) in order to remove ceramides and potential traces of non-cleaved glycolipids [13]. To achieve the optimal digestion conditions, different amounts of ceramide glycanase and glycolipids were tested and evaluated with TLC and anisaldehyde staining of the ceramide fraction [10]. The yield was estimated to be more than 90%. The oligosaccharide fractions eluted in the water fraction were lyophilised and permethylated [14] before non-selective GC and GC/MS analysis.

Glycolipid analysis

TLC was performed on HPTLC plates (Si-60; Merck, Germany and HP-KF; Whatman, UK) using the solvent chloroform/methanol/water, 60/35/8 (by volume). 5–10µg of the GSL mixtures was applied to the HPTLC plate. Detection was accomplished with the anisaldehyde reagent [10] or with alkaline phosphatase conjugated secondary MAbs according to a modification of the method of Magnani *et al.* [15, 16] and the method of Hynsjö *et al.* [17]. The primary identification of the glycosphingolipids was based on the migration and antibody staining on the HPTLC plates. The MAbs and reference glycosphingolipid fractions used in the thin layer immunostaining assays are presented in Table Ia and b.

Glycolipid preparation for NMR analyses

About 3.5 mg (estimated amount of approximately 300µg of each component) of each glycosphingolipid fraction was deuterium-exchanged in excess of $CDCl_3/CD_3OD$ (2:1 by volume). The LC-NMR samples were dried and dissolved in 100µL of $CDCl_3/CD_3OD/D_2O$ in the proportions 80/20/1 (by volume) for fraction *i,* and 65/25/4 (by volume) for fraction *ii.* Samples used for conventional ^1H NMR analyses were dissolved in DMSO.

Liquid chromatography-nuclear magnetic resonance

A gradient of $CDCl_3/CD_3OD/D_2O$ (Dr. Glaser AG, Switzerland) in the proportions 80/20/1 (by volume, solvent A) to 60/35/8 (by volume, solvent B) was applied for the non-polar fraction (*i*). A mixture of $CDCl_3/CD_3OD/D_2O$ in the proportions 65/25/4 (by volume, A) to 40/40/12 (by volume, B) was applied for the polar fraction (*ii*). A constant flow rate of 0.5 mL/min. was used over 280 min. The rendered fractions from the LC "on-flow" NMR where pooled and analysed by conventional ^1H NMR.

An LC33 UV-detector operating at 235 nm was interfaced to the LC and the NMR probe head was used to indicate the composition of the components compounds.

The LC 22C pump was equipped with a straight phase silica column (250 × 4.6 i.d., Spherisorb, UK) and connected on-line to the Bruker AMX 500 MHz spectrometer with an X32 acquisition system. The spectrometer was equipped with a dedicated ^{1}H flow probe (4.0 mm. i.d.) with a cell volume of 160 µL. Continuous flow ^{1}H NMR spectra were obtained using a one dimensional NOESY (nuclear Overhausen effect spectroscopy) pulse sequence including presaturation. An acquisition time of 1 s was used with a recycling time of 1.5 s, giving a time resolution of 48 s/increment. The spectral line width was 6 kHz with a time domain of 12 k. The pseudo-2D spectra were obtained using a sine-squared window multiplication prior to Fourier Transformation in the f2-dimension. Chemical shifts were referenced to the methyl-group in methanol at δ 3.31. Presaturation frequencies were achieved by reaching equilibrium with solvent A before and after each experiment. Blank runs were performed for each experiment without any solvent suppression using a pulse programme to simulate pulses and time delays of the NOESY experiment. Frequency values for the suppression were read from these lists. The NMR acquired dates for 40 min., with a complete chromatography being completed in 50 min. All experiments were performed at room temperature (20–22°C).

High-temperature capillary gas chromatography

Fused silica capillary columns (10m × 0.25 mm i.d., Chrompack, The Netherlands) were D4-deactivated and statically coated with 0.03 µm of PS 264 (Fluka, Switzerland) which was cross-linked using dicumyl peroxide [18]. Capillary GC was performed using a Hewlett-Packard 5890-II gas chromatograph equipped with an on-column injector and a flame ionization detector (detector temperature 400°C). Hydrogen was used as the carrier gas, with an oxygen trap in the carrier gas line and a head pressure of 0.7 bar, giving an average linear gas velocity of 125 cm/s at 70 °C. The permethylated oligosaccharides were dissolved in 200 µL of ethyl acetate and 1 µL was injected on-column at 70 °C (1 min.) then programmed up to 200 °C at 50 °C/min. followed by 10 °C/min. up to 400 °C.

High-temperature gas chromatography–mass spectrometry

GC/MS was performed on a Hewlett-Packard 5890-II gas chromatograph interfaced to a JEOL SX-102A mass spectrometer (Jeol, Japan). The gas chromatograph was equipped with an on-column injector which was under electronic pressure control. Helium was used as the carrier gas, with an oxygen trap in the carrier gas line and with a head pressure of 0.3 bar at the start, giving an average linear gas velocity of 87 cm/s at 80 °C. GC/MS was performed with the on-column injector in constant-flow mode (1.9 mL/min) and vacuum compensation on with the electronic pressure control. The fused silica column tip was positioned in the ion source about 2 mm from the electron beam. Gas chromatographic conditions: fused silica column (10 m × 0.25 mm i.d.) coated with 0.03 µm of cross-linked PS 264 (Fluka, Switzerland) 1 µL of sample dissolved in ethyl acetate was injected on-column at 80 °C and the same two-step temperature programme as described above for GC was used [19].

Mass spectrometry conditions: acceleration voltage + 10 kV; electron energy 70 eV; trap current 300 µA; ion source temperature 360 °C; GC/MS interface temperature 380 °C; linear magnet scan and mass range scanned *m/z* 100-1600; total cycle time 1.4 s; resolution 1400 (m/Δm, 10% valley definition); pressure in the ion source region $5 \cdot 10^{-4}$ Pa.

Matrix-assisted laser desorption/ionization mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed on a TofSpec-E time-of-flight mass spectrometer (Micromass, England) in the positive reflectron mode at +22.5 kV acceleration voltage. A thin-film matrix surface was prepared using the fast evaporation technique from 2,5-dihydroxybenzoic acid (16.2 mg/mL in acetone containing 10 mM LiCl). The permethylated oligosaccharides were dissolved in 200–500 µL of ethyl acetate and 0.5 µL was applied to the matrix surface. The lithium adducts of the molecular ions $[M + Li]^+$ were observed.

Results

Glycosphingolipid components

The extensive use of silicic acid columns during the basic preparation process (see Materials & method) caused a successively increased loss of longer glycosphingolipid components (e.g. pentaglycosylceramides and longer) from the fraction, due to irreversible binding of GSL to the silicic acid. This occurred despite the fact that the column was eluted with chloroform/methanol/water in the proportions 40/40/12 (by volume) as a finishing step after each silicic acid column run. MAbs detected only traces of longer GSLs after the total preparation was finished.

Thin layer immunostaining

The anti-P^k MAb and the anti-P MAb showed positive staining in the three and four sugar region respectively (not shown), indicating the presence of globotriaosylceramide (Gb_3Cer) and globotetraosylceramide (Gb_4Cer).

The anti-nLc_4 antibody IB_2 failed to detect the neolactotetraosylceramide (nLc_4Cer) (type 2 chain) glycolipid in the mixture (not shown) but it was later detected with NMR and GC/MS.

Positive staining in the five sugar GSL region was seen with the anti-H type 2 antibody Chembiomed (not shown)

Table 1a Monoclonal antibodies used for thin layer immunostaining.

Antibody	*Specificity*	*Code no.*	*Reference*
Anti-A	A determinants, all types	Dakopatts A581, Denmark	[29]
Anti-B	B determinants, all types, Galα1,3Galβ terminal	Dakopatts A582, Denmark	[29]
Anti-H	H determinants, type 1 chain	Dakopatts A583, Denmark	[29]
Anti-H	H determinants, type 2/3 chain	Lot 9BH R0001 Chembiomed, Canada	
Anti-Le^a	Le^a terminal	Seraclone	
Anti-Le^b	Le^b terminal and H type 1	Seraclone	
Anti-P^k	P^k terminal		*
Anti-P	Gb_4Cer	CLB ery-2	[30]
Anti-Galα1,3Galβ	Galα1,3Galβ terminal, type 2 chain	TH-5	**
Anti-Galα1,3Gal	Galα1,3Galβ terminal, type 2 chain	P3393	***
Anti-A type 3/4	A determinants, type 3/4 chains	HH-5	[31]
Anti-A type 3	A type 3	TH-1	[32]
Anti-$Le^{b/H}$	Mono- and di-fucosyl, type 1 chain	075 clone LM129/181, ****	[33]
Anti-$Le^{a/c}$	Mono- and di-fucosyl, type 2 chain	069 Clone BRIC87, South West Reg. Transf. Centre, UK.	[33]
Anti-Forssman	Forssman antigen	MAS 033B, Sera-lab, UK.	[34]
Anti-A type 1	A type 1, monofucosyl	AH-21	[35]
Anti-A type 2	A determinants, type 2 chain	HH-4	[31]
Anti-nLc_4	Tetraglycosylceramide, type 2 chain	IB_2	[36]
Anti-Gg_4	Ganglio series	AG_1 Seikagaku Inc., Japan	[37]

*Proceedings of the Second Workshop and Symposium on Monoclonal Antibodies against Red Cells and Related Antigens.(1990) *J Immunogenet.* **17:**350.
**Thorn, J., Hakomori, S., Clausen, H., *unpublished results.*
***Strokan, V., Mölne, J., Svalander, C., Breimer, M E., *in press.*
****Glasgow & West Scotland Blood Transfusion Service Law Hospital,UK.

and the anti-$Le^{b/H}$ antibody (075 clone LM129/181) (not shown). The 7/8 sugar region also stained positive for the Chembiomed Anti-H MAb. Dakopatts anti-H MAb stained negative (Table 1a) (not shown).

Two different α-Galactose (Gal) MAbs (TH-5, P3393) were used to confirm the presence of the Galα1,3Gal epitope. Both antibodies showed binding in the 5 sugar region, probably to the Galα1,3nLc_4Cer, but also to more slowly moving glycolipids (7/8, 10/12 sugars) (Fig. 1b and c respectively). The TH-5 antibody revealed binding in the 6 sugar region in the pig lung fraction. Dakopatts anti-B antibody showed positive staining for the polar pig lung fraction with binding in the 5 sugar region. The anti-B antibody has earlier been shown to react with the defucosylated "B-like" epitope expressed on the Galα1,3nLc_4 structure [3].

The different anti-A antibodies used (Dakopatts, AH-21, HH-5) are included in Table 1a. Other MAbs used were anti-Le^a (Seraclone), anti-Gg_4 (Seikagaku Inc.), anti-$Le^{y/b}$ (Dr. Fraser), $Le^{a/c}$ (Dr. Anstee), anti-Forssman and anti-nLc_4 monoclonal IB_2 (not shown) (Table 1a).

Nuclear magnetic resonance analyses

Separation and pooling of fractions were monitored by α- and/or β- anomeric signals. Individual components were identified by further spectral identification where LC-NMR spectral data were validated against conventional ^1H

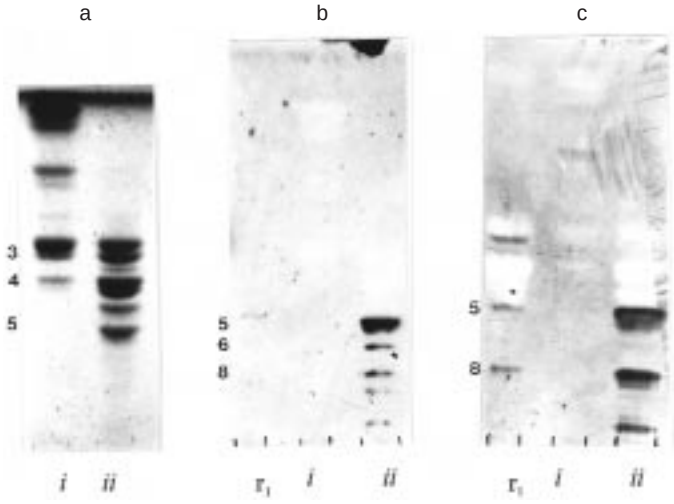

Figure 1. TLC and immunostainings with MAbs of glycolipid fractions *i* and *ii*. 1a. Thin layer chromatogram (TLC) of glycolipid fractions *i* (fast moving fraction with ≤ 3 sugar residues in the carbohydrate chain) and *ii* (slow by moving fraction with ≥ 3 sugar residues in the carbohydrate chain) from 4 pig lungs. 2μ of each fraction was added to the HPTLC plate and chromatographed in chloroform/methanol/water in the proportions 60/35/8 (by volume). After drying, staining was accomplished with the anisaldehyde reagent. The numbers of sugars in the carbohydrate chains are indicated to the left in figs a–c. 1b. Thin layer immunostaining with the anti-Galα1,3Galβ antibody TH-5. 10 μg of a total pig aorta fraction is used as reference (r_1). The anti-Galα1,3Galβ antibody P3393 is used for the thin layer immunostaining in 1c. The reference is the same as in 1b.

Table 1b Glycolipid references used for Thin layer immunostaining.

References	*Origin*	*Specificity*	*Reference*
A-6-1	Human A_1 erythrocytes	Blood group A hexaglycosylceramide type 1 chain	[38]
B-6-1	Human A_1B $Le^{(a-b-)}$ pancreas	Blood group B hexaglycosylceramide type 1 chain	*
H-5-1	Pig kidney	Blood group O pentaglycosylceramide type 1 chain	[39]
H-5-2	Human blood group O erythrocytes	Blood group O pentaglycosylceramide type 2 chain	[40,41]
Galα1,3nLc_4Cer	Pig aorta	Galα1,3nLc_4Cer	[28]
P^k	Human erythrocytes	P^k (globotriaosylceramide)	[42]
P	Human erythrocytes	P (globotetraosylceramide)	[42]
Gb_4Cer	Human kidney	Blood group A tetraglycosylceramide type 4 chain	[43]
Le^b	Human small intestine	Difucosyl type 1 chain hexasaccharide	[44]
Le^a	Human small intestine	Monofucosyl type 1 chain pentasaccharide	[44,45]
Forssman	Dog intestine	The Forssman hexaglycosylceramide	[46]
A-7-1	Dog intestine	Blood group A septaglycosylceramide type 1 chain	[47]
A-7-2	Human A_1 erythrocytes	Blood group A septaglycosylceramide type 2 chain	[38]
A-9-3	Human kidney vein	Blood group A nonaglycosylceramide type 3 chain	[43]
nLc_4Cer	Dog small intestine	Tetraglycosylceramide type 2 chain	**
Gg_4Cer	Mouse intestine	Gangliotetraosylceramide (ganglio series)	[48]

*Bäcker, A. E., and Holgersson, J., unpublished results
**Samuelsson B. E et al. unpublished results

NMR spectra of separated fractions in comparison with reference NMR data [5,6,20,21]. This process of validation of LC-NMR spectral data by comparison with conventional ^{1}H NMR data on isolated fractions increased the structural predictability of LC-NMR in future analysis. The f1 dimension in pseudo-2D spectra can be labelled in two ways, as "rows", which refers to the order of a given spectrum in the series of spectra arrayed along this axis, or as time (tr). We used time and the symbol "tr" since time is similar to the LC retention time.

The continuous flow LC-NMR analysis of the fast moving fraction *i* allowed separation and identification of three major components. The first solute had a retention time tr of 15.52min (row 21, Fig. 2a, 2D plot). The corresponding ^{1}H-NMR spectrum displayed one β-anomeric signal at 4.18ppm (J=7.72Hz), as expected for the Glcβ1 in monoglycosylceramide (Fig. 2b).

The proton NMR spectrum from row 25 at tr=19.36min gave rise to two β-anomeric resonances at 4.20ppm (J=7.72Hz) and 4.27ppm (J=7.72Hz). The resonances are characteristic for the Glcβ1 and Galβ4 originating from the lactosylceramide (LcCer) (Galβ1, 4Glcβ1, 1Cer) (Fig. 2c).

The third component, eluted at tr=24.32min (row 31), was identified as Gb_3Cer e.g. Galα1, 4Galβ1, 4Glcβ1, 1Cer. The relevant proton NMR spectrum exhibited two β-anomeric protons at 4.21ppm (J=7,72Hz) and 4.34ppm (J=7.35Hz) which were consistent with a lactosyl core and an additional α-anomeric peak resonating in the Galα frequency domain (4.89ppm, J=3.67Hz) (Fig. 2d). The downfield shift of 0.07ppm observed in Galβ4 in the glycosylated lactosyl fragment is note worthy.

The LC "on-flow" NMR analysis of the slowly moving fraction *ii* revealed the presence of four different glycolipid components (2D plot, Fig. 3a). The numbering of the components in the NMR spectra is identical to the numbering in the gas chromatogram in Figure 4, and in the TIC in Figure 5a.

Component 1, Gb_3Cer, was present also in this fraction (not shown). The fraction was dominated by component 3, a four sugar glycolipid eluted approximately from tr=18.60min (row 30) to tr=25.82min (row 41). A specific NMR profile of the relevant solute is given in Figure 3b. Three β-anomeric signals at 4.22ppm (J=7.72Hz), 4.35ppm (J=7.35Hz) and 4.51ppm (J=8.09Hz) were assigned to Glcβ1, Galβ4 and N-acetylgalactoseamineβ3 (GalNAc) respectively. The structure was completed by one α-anomeric proton at 4.83ppm (J=3.68Hz) which corresponded to Galα4. The component exhibited the features of Gb_4Cer.

Two other slow-moving glycolipids followed Gb_4Cer. The first, component 5, was detected at row 43 at tr=26.83min (Fig. 3c). It contained one α-fucosyl unit resonating at a typical chemical shift of 5.10ppm. The second glycolipid (component 6) was detected at row 49 at tr=30.63min (Fig. 3d). The proton NMR spectrum of row 43 revealed four anomeric signals. According to previous work and immunological methods such as thin layer immunostaining, the expected fucose-containing glycolipid in the fraction was a pentasaccharide-based glycolipid. The occurrence of a masked glycosylated Galβ4 peak at 4.34ppm relative to a glycosylated lactosyl fragment was assumed and later proved in the current run of Gb_3Cer and Gb_4Cer (Fig. 3d). Glcβ1 and GlcNAcβ3 appeared at typical reso-

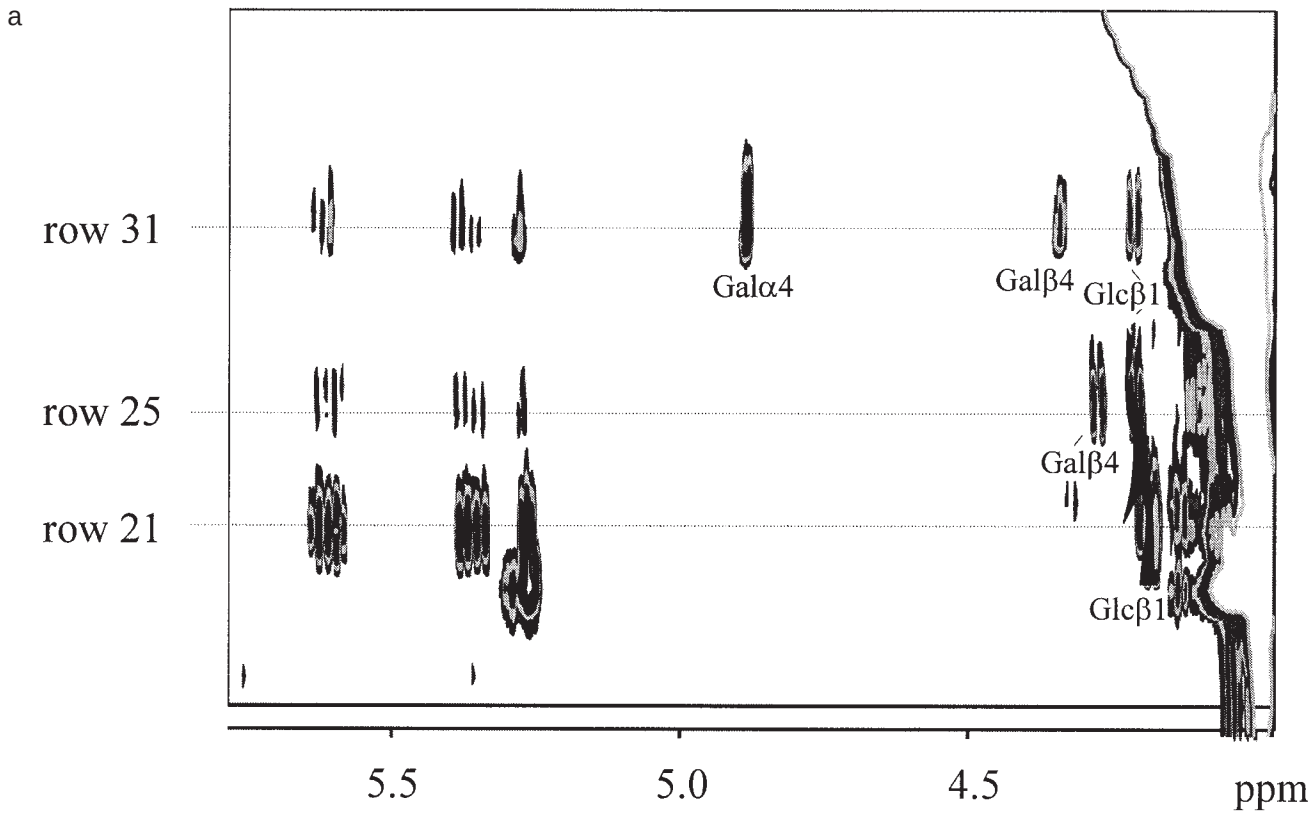

Figure 2. 2D plot and 1D spectra of fraction *i.* 2a. LC-NMR 2D plot of fraction *i.* All spectra obtained from this experiment are shown in the 2D plot. To the left the chromatography and dynamics of the gradient are presented. The y-axis is given as rows, which increase with the time of the gradient. Short sugar chain components are eluted early and are found in the lower part of the plot. Larger, more polar components, eluted later and are found at the top. The chemical shifts are marked on the x-axis. The 2D plot shows in row 31 the globotriaosylceramide, in row 25 the lactosylceramide and in row 21 the monoglycosylceramide. Fig 2d–b show the 1D spectra of the 3 components globotriaosylceramide (2d), lactosylceramide (2c) and monoglycosylceramide (2b). The different anomeric protons in the sugar part of the glycolipids and are indicated in the spectra. The 1D spectra are extracted from the 2D plot (rows 31, 25 and 21).

nances of 4.22ppm and 4.57ppm respectively. The β-anomeric proton at 4.28ppm (J=7.72Hz) was assigned to be the Galβ4 in position 4 in the carbohydrate core saccharide. The component was interpreted as the H-5-2 glycolipid.

The presence of a Galβ4 at 4.34 ppm was taken into account in the characterisation of component 6), interpreted to be Galα1,3nLc_4Cer. The Galβ4 resonance was hidden by an intense solvent peak. The α-anomeric signal assigned to Galα3 arose at 4.95ppm (J=2.94Hz) in addition to the four peaks from the earlier characterised nLc_4Cer.

The LC "on-flow" NMR-rendered fractions were pooled and characterised by conventional ^{1}H NMR. Most of the spectra showed pure components or no more than two different components in the same test tube. The spectra were interpreted and signals found to be in agreement with the LC "on-flow" NMR results (not shown).

Gas chromatography-mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry analyses

The TIC from GC/MS and the molecular weights obtained from MALDI-MS analyses of the corresponding glycanase released, permethylated oligosaccharides are shown in Figure 5 and Table 2. The peak numbers in the TICs correlate to a specific glycosphingolipid whose structure is given in Table 2. The gas chromatogram of glycanase-released oligosaccharides from pig lung fraction *ii* was consistently reproduced in the TIC from GC/MS as assessed by the conserved scan times and peak intensities (Fig. 4 and 5a). Mass spectra of special interest were selected from the GC/MS analysis and are shown in Figure 5b-c (see below). The EI^+ mass spectra of permethylated oligosaccharides contained sequence oxonium ions (B_i ions) including the non-reducing end of the saccharide chain. In the presence of a N-acetylhexoseamine (HexNAc), fragment ions (Z_i

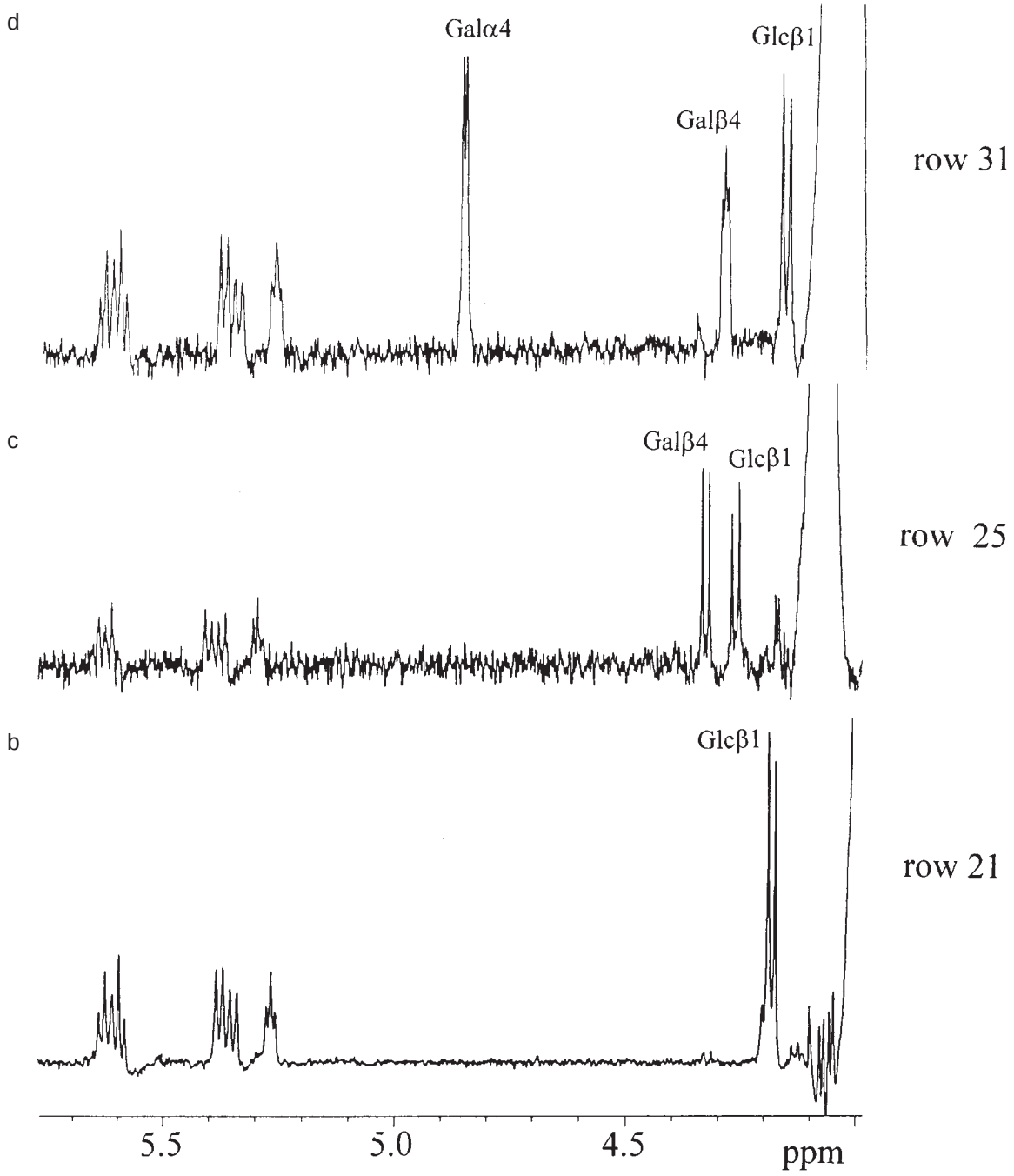

Figure 2. (Continued)

ions) [22] including the reducing end of the saccharide chain and arising from an inductive cleavage of the glycosidic bond at the non-reducing end of the HexNAc residue appeared. Together, these complementary ions gave the complete sequence of the oligosaccharide in most cases.

The capillary GC of fraction *ii* gave qualitative and semiquantitative estimates of the oligosaccharide components found in the sample mixture obtained after ceramide glycanase cleavage of the glycolipid fraction. Estimations of number of sugars in the oligosaccharide chains were based on their retention time on the column and earlier experiments [3]. The result are presented in Figure 4. The numbering of the components in the GC chromatogram in Figure 4 is identical to the numbering in the NMR spectra in Figure 3, and the total ion chromatograms (TICs) in Figure 5a.

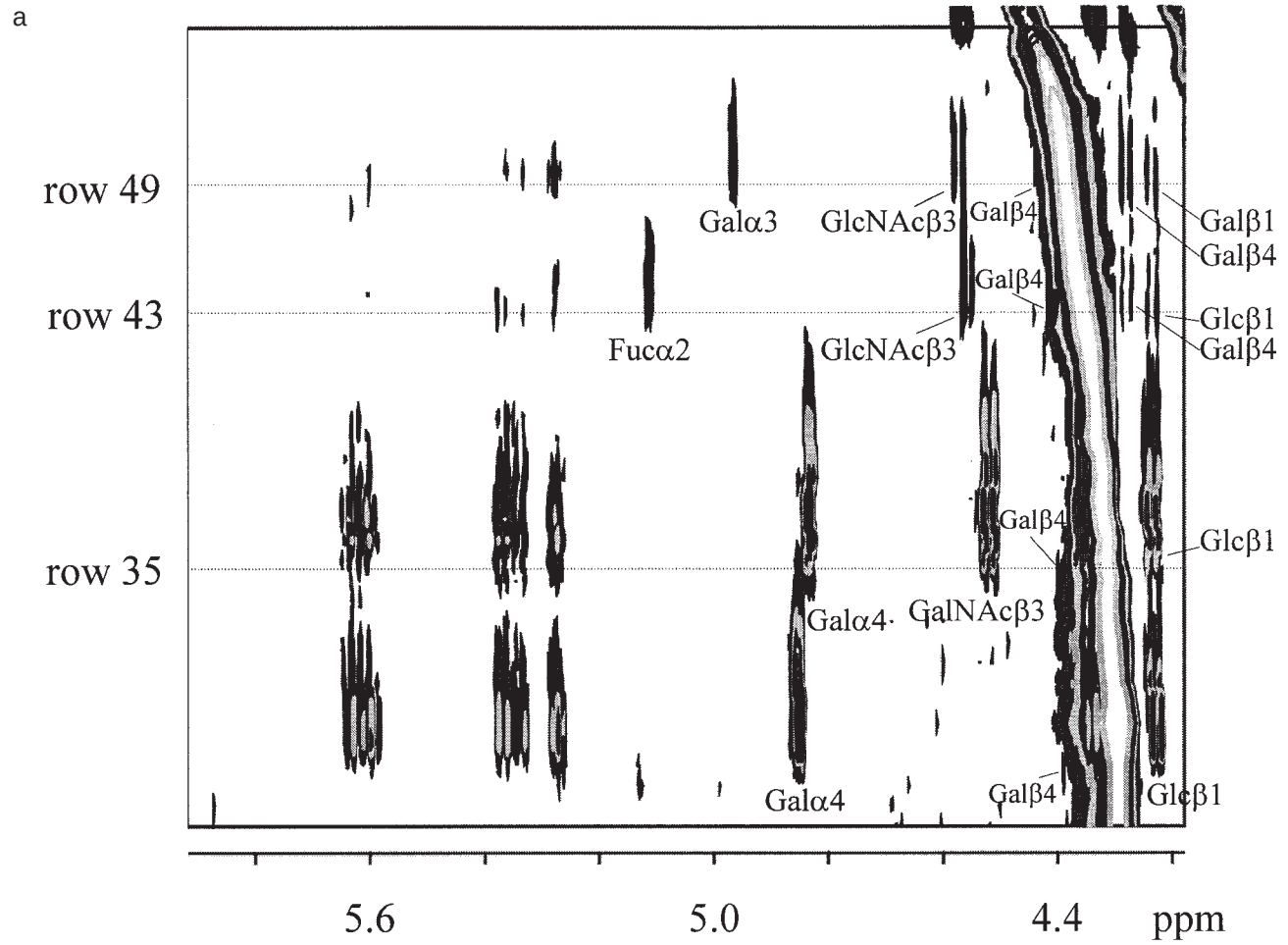

Figure 3. 2D plot and 1D spectra of fraction *ii.* 3a. LC-NMR 2D plot of fraction *ii,* with rows/time on the y-axis and chemical shifts indicated on the x-axis (as in Fig. 2a). Row 49 shows the Galα1,3neolactotetraosylceramide component, row 43 the H-5-2. The last row, 35, indicates the globotetraosylceramide component. 3d–b shows extracted 1D spectra from the 2D spectra in figure 3a. Galα1,3neolactotetraosylceramide is found at row 49 (3d), H-5-2 at row 43 (3c), and the globotetraosylceramide is found at row 35. The anomeric protons are indicated in the spectra.

The mass spectrum of component 1 in Figure 5a indicates a trisaccharide structure (M= 658.4 Da). Oxonium ions at *m/z* 219 (B_1), ***m/z*** 423 (B_2) gave the sequence Hex-O-Hex- from the non-reducing end of the saccharide, and the same fragment ions also indicated a reducing end sequence containing a -Hex-O-Hex, thereby completing the sequence of Gb_3 as given in Table 2. From its spectrum (not shown), component 3 (Fig. 5a) was identified as a HexNAc-terminated tetrasaccharide (M=903.5 Da). The sequence HexN-O-Hex- was determined from the oxonium ions at *m/z* 260 (B_1) and ***m/z*** 464 (B_2) and identified as the Gb_4 oligosaccharide (Table 2).

The nLc_4 oligosaccharide was identified in the spectra (not shown) of component 4 (Fig. 5a). The intense fragment ion *m/z* 182 revealed the type 2 chain and excluded the Lc_4 oligosaccharide [3]. Oxonium ions at *m/z* 219 (B_1), ***m/z*** 464 (B_2) and ***m/z*** 668 (B_3) gave the Hex-O- HexN-O-Hex- structural terminal. The reducing end structure -HexN-O-Hex-O-Hex was shown by the fragment ion at *m/z* 668 (Z_3).

Component no. 5 in the TIC (Fig. 5a) was shown to be a pentasaccharide with a molecular mass of M=1077.6 Da (Fig. 5b). The sequence dHex-O-Hex-O-HexN-O-Hex was shown by the fragment ions at *m/z* 189 (B_1), *m/z* 393 (B_2) *m/z* 638 (B_3) and *m/z* 842 (B_4). The reducing end structure (Hex-O-HexN-O-Hex-O-Hex) was determined by fragment ions at *m/z* 668 (Z_3) and *m/z* 872 (Z_3). The H-5-2 structure could be differentiated from its isomer H-5-1 on the basis of differences in retention times and in intensity ratio between the fragment ions at *m/z* 182 and *m/z* 228. In the presence of an internal carbon 4-substituted GlcNAc, the ration is very high, the ion at *m/z* 182 often being the base peak [3,23,24]. Components containing an internal 4-substituted GlcNAc elute in front of the isomer with a 3-substituted GlcNAc, which has been shown earlier for

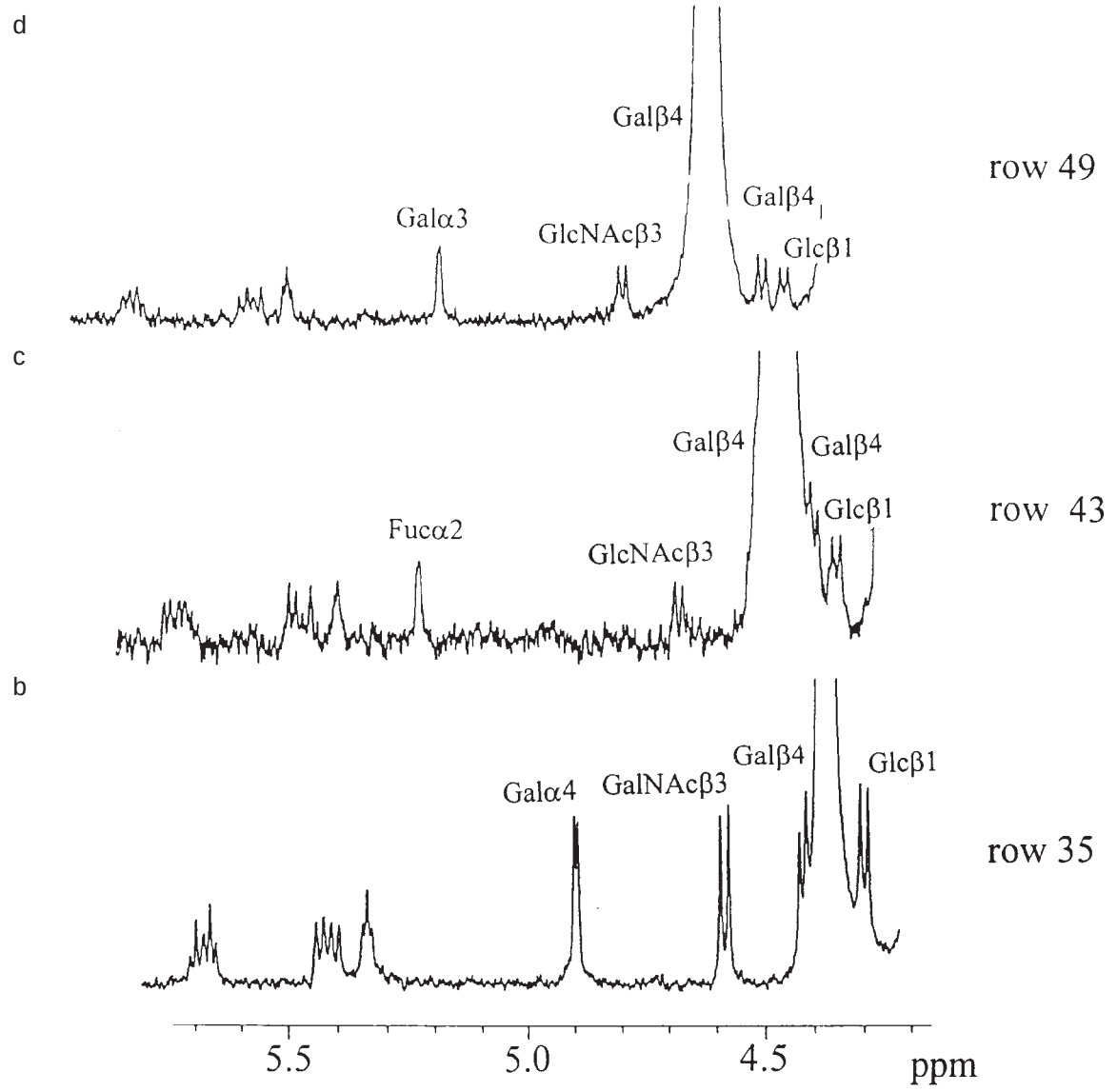

Figure 3. (Continued)

lactotetraosylceramide; (Lc_4Cer) (type 1 chain) and nLc_4Cer (Teneberg & Karlsson, unpublished results), and A-6-2 and A-6-1 [23,25].

In the mass spectrum of component 6 (Fig. 5c) a Hex-O-Hex-O-HexNAc- sequence was indicated from the oxonium ions at *m/z* 219 (B_1), ***m/z*** 423 (B_2) and ***m/z*** 668 (B_3). The structure from the reducing end (-HexN-O-Hex-O-Hex) was given by the inductive cleavage at the non-reducing end of the HexNAc residue by the fragment ion at *m/z* 668 (Z_3). The B_4 and Z_4 fragment ions at *m/z* 872 could be identified in the spectrum. The sequence of the pentasaccharide (M=1107.6) is consistent with the Galα3-Galβ4GlcNAcβ3Galβ4Glcβ structure (Galα1,3nLc_4), which presence in the lung fraction was also detected by the two anti-Gal antibodies in the thin layer immunostaining experiments (Fig. 1a and b). All components were detected in the TIC as well as in the gas chromatogram (cf. Fig. 4, 5a).

Discussion

Sensitivity

The LC "on-flow" NMR facilitates GSL characterisation and may be used for purification and pooling purposes in the screening of mixtures.

The sensitivity was evaluated and compared with that of conventional ^1H NMR and GC/MS. LC-NMR demonstrated less sensitivity compared to conventional NMR, basically due to the reduced signal sampling time. Sampling

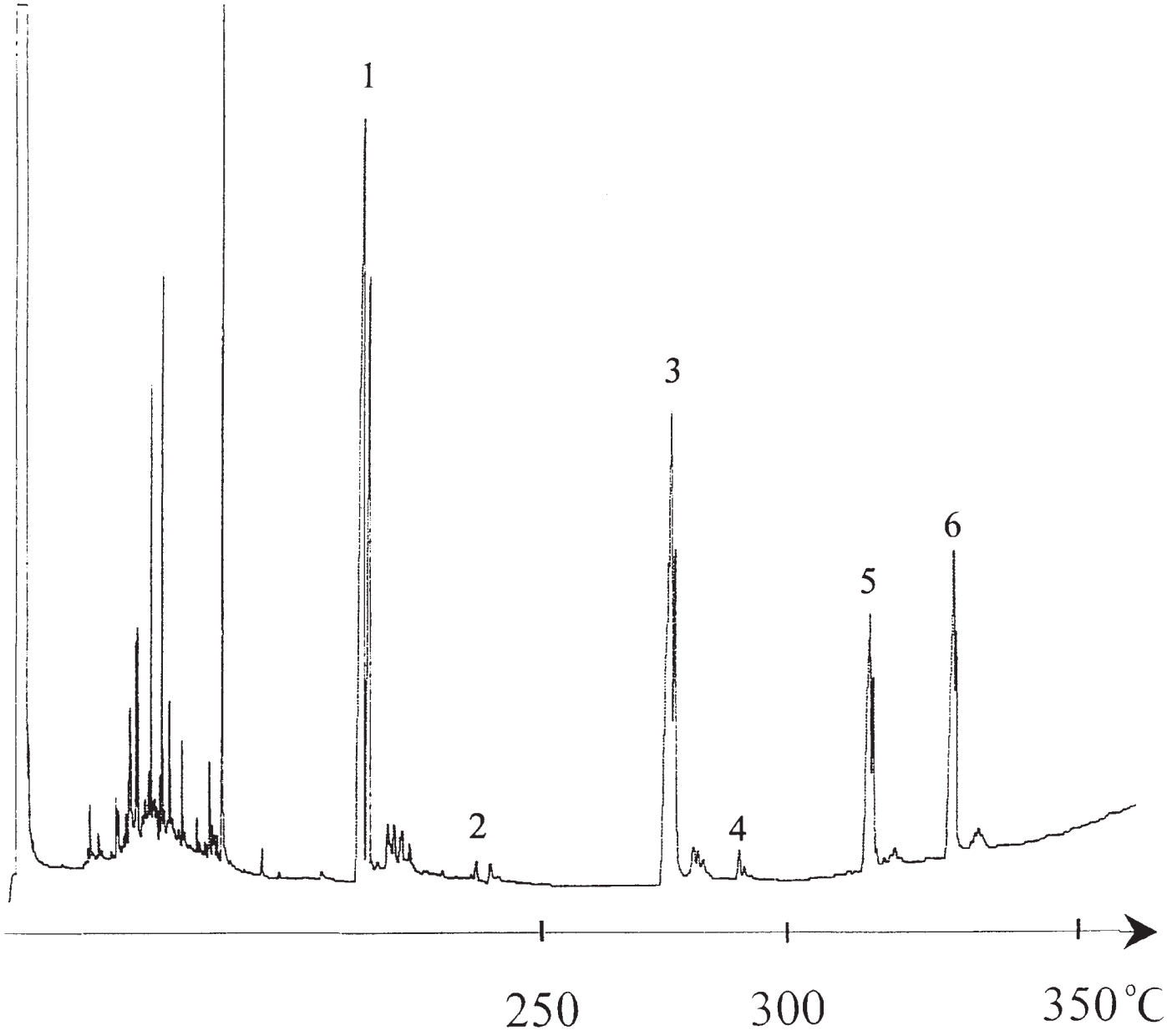

Figure 4. Gas chromatogram of fraction *ii* (to be compared with TIC of the fraction in Fig. 5a for reproducibility). Six components were detected in the gas chromatogram, starting with two trisaccharide components (nos. 1 and 2), two tetrasaccharide components (nos. 3 and 4) and two pentasaccharide components (nos. 5 and 6).

time and sensitivity could be further increased by using the "stop-flow" mode. Just comparing detection sensitivity, GC/MS on ceramide glycanase-released oligosaccharides is the most sensitive method, needing only a small fraction (1–10 picomole/component) compared to the injected amount of 300μg for the LC-NMR analysis. The mass spectra are of high quality and on single component structures due to the chromatography, which facilitates interpretation of the oligosaccharide. The mass spectra showed characteristic and easily interpretable fragmentation since the fragmentation occurred from both ends of the oligosaccharides, which favoured oligosaccharide symmetry.

The GC/MS sensitivity was illustrated by the detection of the nLc_3 and the nLc_4 oligosaccharide. In the total ion chromatogram (TIC), the components were easily recognised despite low abundance. Both oligosaccharides were identified in the mass spectra, nLc_3 by its intense terminal HexNAc. The LC-NMR failed to recognise Lc_3Cer in the mixture, but could detect nLc_4Cer. Lc_4Cer is only slightly more abundant (Fig. 4,5a), indicating possibilities of LC-

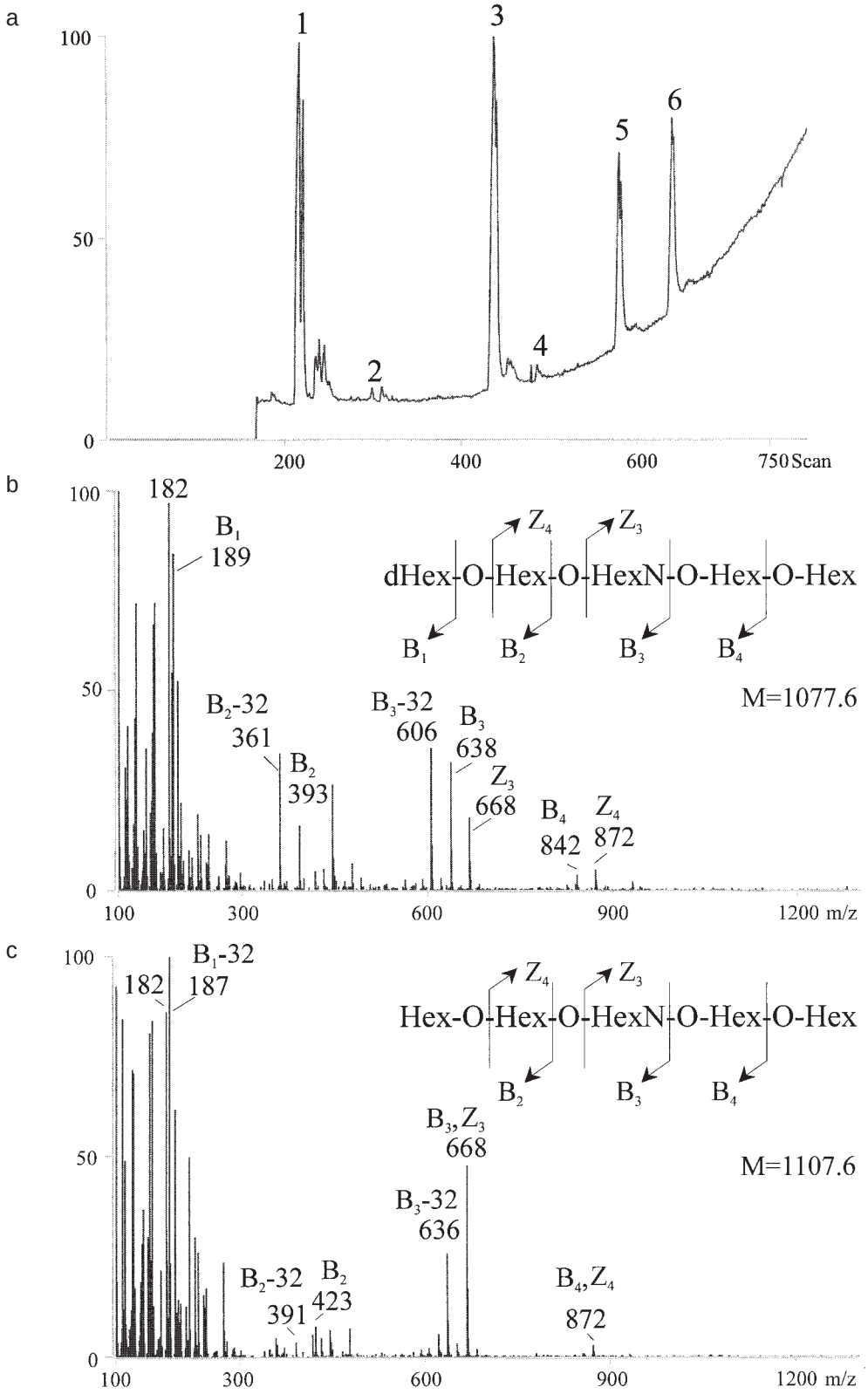

Figure 5. 5a TIC from GC/MS of permethylated oligosaccharides released from glycolipids of pig lung fraction *ii.* The different components are indicated by numbers identical to the numbering in Figure 4. 5b and c shows the mass spectra of component 5, the H-5-2 glycolipid (M = 1077.6) (top), and component 6, Galα1,3nLc_4Cer (M = 1107.6) (bottom). Component 6 shows its characteristic feature with the *m/z* 668 for the B_3 and Z_3 fragments due to the symmetry of the oligosaccharide.

Table 2. Oligosaccharide structures found in fraction *ii* from 4 pig lungs characterised with MALDI-MS.

TIC peak	*Name*	*Structure*	*M (Da):*
1	Gb_3	Hex-O-Hex-O-Hex	658.4
2	nLc_3/Lc_3	HexN-O-Hex-O-Hex	699.4
3	Gb_4	HexN-O-Hex-O-Hex-O-Hex	903.5
4	nLc_4	Hex-O-HexN-O-Hex-O-Hex	903.5
5	H-5-2	dHex-O-Hex-O-HexN-O-Hex-O-Hex	1077.6
6	Galα1,3nLc_4	Hex-O-Hex-O-HexN-O-Hex-O-Hex	1107.6

NMR detection. The finding of Lc_4Cer showed that less abundant components can be characterised and analysed just by the addition of chromatographic separation in LC-NMR. Compared to ^{1}H NMR analysis of the total GSL mixture, the signals from less abundant GSLs were difficult to recognise in the total mass of signal information. Variation in quantity of components is frequently found in biological samples. When smaller sample amounts (200µg) were injected, the majority of the components could still be detected.

Chromatographic separation

The analytical resolution is more dependent on the NMR cell volume and less dependent on the separation over the column [26].

The choice of gradient is determined by the GSL composition in the mixture. Great variation in chain length with long sugar chains required a gradient with a large polarity range for separating these compounds. This in turn necessitated a longer gradient time. The solvent flow rate, gradient composition and chromatography time needed to be optimised for the best resolution. The solvent peak migration towards 4.5 ppm was one side-effect of the polar eluents used. Hydroxyl protons and water decreased the OH bonds in the eluent. A solvent peak at 4.25–4.35 ppm appeared in a critical part of the spectra, which masked the Galβ4 signal in some cases (e.g. Galα1,3nLc_4Cer). The water signal at 4.72 ppm caused disturbances in the spectra, as did the methyl-group of methanol at 3.31 ppm and the hydroxyl-group of methanol at 4.79 ppm. Deuterated solvents were used in all experiments due to susceptibility problems and drift of signals relative to the lock. This increased the experimental costs. By testing other eluents, we hope to be able to diminish the solvent peak migration in critical areas in the future. Improvements in the spectrometer dynamic range of the NMR were described to reduce the need for deuterated solvents in other solvent system [27], but were not fully applicable in our gradient systems. The presaturation position needed to be established for each time slice due to chemical shift changes by the OH bonds, which made autosuppression of solvent peaks difficult.

A UV/visible detector is unsuitable for detecting non chromophores (e.g. GSL) due to the weak absorbance. A detector able to detect GSLs regardless of concentration or solvent composition would make it possible to detect separate GSLs and allow analysis in "stop-flow" mode.

The technique could be further improved by reducing the sample volume and optimising the NMR parameters such as the probe size (sensitivity vs. distance along the cell with a linear field gradient), detection volume and sensitivity of the LC probe (unsealed standard sensitivity solution) [26]. The cell size can be treated as proportional to the cell volume—a large cell gives higher sensitivity, but a decreased chromatographic resolution. This fact must be considered together with the volume of the chromatographic peak, probe design and cell design.

The GSL preparational work can thus be monitored by the non sample-consuming LC "on-flow" NMR technique instead of the commonly used TLC assay. TLC is based on GSL migration in organic solvents on a HPTLC plate. Visualisation of all non-acid GSLs is accomplished with for example, anisaldehyde. GSL migration depends on sugar chain length, ceramide differences and the sterical conformation of the sugar chain (e.g. sugar binding positions and number of side chains). The assay is not capable of distinguishing between isomers such as type 1 and type 2 chain based structures. A single glycolipid component separates slightly different with different ceramide compositions and gives rise to two or more distinct bands on the TLC. Two different glycolipids with the same number of sugar residues can easily be misinterpreted as one component and can be incorrectly pooled together.

The TLC assay can be made more specific by the use of carbohydrate-specific MAbs for visualisation (thin layer immunostaining). The technique is used for screening of epitopes of special interest. However, only one specific MAb can be used at a time, only the Mab-specific part of a GSL is recognised and only well-characterised antibodies can be used. The complete structure of a GSL is not revealed by thin layer immunostaining.

In the case of LC-NMR, the silica column separates GSLs on the basis of sugar chain length, ceramide differences and the sterical conformation of the sugar chains (as for the TLC plate), but it also introduces a solvent gradient

in the column as an extra separation factor. This makes it possible to separate one part of the GSL fraction efficiently by using solvent gradients, which through polarity separate the chosen region of the GSL mixture (for example polar solvent gradients efficiently separate the more polar GSLs in a mixed fraction while less polar gradients separate less polar GSLs in the mixture). Separated GSLs are introduced "on-flow" into the NMR magnet, and the established spectra show binding positions and α- and β anomeric protons of each introduced component.

Comparing TLC and LC-NMR, the advantages of the LC-NMR are the extra separation dimension by solvent gradients and the more detailed structural information. The disadvantages of LC-NMR are low sensitivity and high cost.

LC-NMR data/^{1}H NMR data

The fractions obtained from the LC "on-flow" NMR experiments can be pooled and used for conventional ^{1}H NMR, which has been done in this study. The conventional spectra have been used as a control and as a validation of the LC-NMR data from earlier screenings. Since the LC-NMR technique has some disadvantages (see above), validation/comparison of the spectra needs to be performed with conventional ^{1}H NMR. After for example signal drift and hidden signals have been taken into consideration for the comparison, the LC-NMR spectra can in turn be used as reference spectra for future studies.

Complementary techniques giving structural specificity

Our objectives over the last few years have been to identify GSLs and characterise the expressed carbohydrate antigens on the surfaces of different pig organs, an animal which may be suitable as an organ donor for xenotransplantation to man. The studies are based on biochemical and immunochemical methods involving glycolipid characterisation by thin layer immunostaining, GC, ^{1}H NMR and mass spectrometry. All techniques require well-characterised and pure reference GSLs, which in turn raises the problem of producing sufficient amounts of pure, single GSL fractions.

By means of LC "on-flow" NMR, single components of a GSL mixture can easily be structurally characterised. At this stage of LC-NMR development for native GSLs, the reproduction of the GSL spectra by conventional ^{1}H NMR of the earlier LC-NMR fractionated and pooled fractions, can be used to confirm the findings from the screening. The spectra are used to evaluate eluent drift in the LC "on-flow" NMR experiments, and can exclude interpretation of fake GSL signalling due to solvent disturbances. It is important to include results from LC "on-flow" NMR, ^{1}H NMR, MALDI-MS, GC, GC/MS and thin layer immunostaining before performing the final structural characterisation of the mixture. The techniques complement each other, a single technique does not give all information necessary for total structural GSL characterisation. It should be pointed out that "structure" in some cases means total 3D-structure, which often necessitates X-ray crystallography and/or extensive NMR analysis. In our setting, NMR shows binding positions and configurations, GC nos. of components/sugars involved in the different oligosaccharides, GC/MS completes the GC with the hexose (Hex) structures of each oligosaccharide, MALDI-MS gives the oligosaccharide molecular weight and thus the total number of sugars, and, finally, the immunostaining shows the MAb binding epitope of the GSL. As different techniques present different kinds of information for the sample, it is easy to understand that the information obtained does not always agree. The thin layer immunostaining illustrates this descripancy. The result from this assay are, in most cases in agreement with the NMR and MS findings. However, the negative staining for nLc_4Cer, which was shown to exist in small amounts by NMR and MS techniques, might be explained by the fact that the amount applied for TLC was not sufficient for the antibody to recognise the epitope. Another possibility is that the dominating gangliotetraosylceramide (Gg_4Cer) inhibit the binding of the anti-nLc_4 antibody.

Glycolipid identity

The findings of Galα1,3nLc_4Cer by LC "on-flow" NMR (Fig.1 c–d), conventional ^{1}H NMR (not shown) GC/MS (Fig. 5a,c) and two anti-Galα MAbs (Fig. 1 c–d) in the pig lung have not been shown earlier, although the presence of Galα1,3nLc_4Cer was expected, due to the fact that the pig has been shown to express this antigenic structure in other organs [3]. The dominance of globo-based glycolipids is also in agreement with other studies on endothelium rich pig tissue, e.g. the aorta [28].

The preparation procedure of GSLs has in this case discriminated longer, less abundant components, which have not been eluted in the expected amount from the columns. By reducing the purifycation steps, the irreversible binding of the GSL to silicic acid can be reduced to a minimum. Work is in progress to characterise the more slow moving GSLs from pig lung, i.e. >5 sugar GSLs.

In this paper, we used the α- and β-anomeric proton resonance obtained with a LC "on-flow" NMR technique to pool and structurally characterise native carbohydrate antigens in a GSL mixture from 4 pig lungs. The LC "on-flow" NMR technique was combined with gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS) and matrix assisted laser desorption/ionisation-mass spectrometry (MALDI-MS) of released permethylated oligosaccharides for the GSL characterisation and validation of LC-NMR data.

Acknowledgements

The study was supported by the Swedish Medical Research Council grant no 6521, K97-17X-00536-33AK and nos. 3967, 10435 (to K.-A. Karlsson), The Immunology Concerted Action (3026PL95004), Biotechnology programme contract no. BI04-CT95-004 of the European Union Biotechnology programme, Shared cost Biotechnology programme PL962242, The Inga-Britt and Arne Lundberg Foundation and Göteborg Dental Society.

Pernilla Sandberg is thanked for technical assistance during the NMR experiments. Dr. Hasse Karlsson's technical assistance and valuable help with GC and GC/MS is gratefully acknowledged. We are greatful to Prof. K.-A. Karlsson for allowing us to use the mass spectrometry equipment. Mattias Olsson is thanked for additional preparational work.

References

1 Samuelsson BE, Rydberg L, Breimer ME, Bäcker AE, Gustavsson ML, Holgersson J, Karlsson EC, Uyterwaal A-C, Cairns TDH, Welsh KI (1994) *Immunol Rev* **141**: 151–68.
2 Bäcker AE, Breimer ME, Samuelsson BE, Holgersson J (1997) *Glycobiology* **7**: 943–53.
3 Bäcker AE, Holgersson J, Samuelsson BE, Karlsson H (1998) *Glycobiology.* **8**: 533–45.
4 Spraul M, Hofman M, Dvorsak P, Nicholson JK, Wilson ID (1993) *Anal Chem* **65**: 327–30.
5 Spraul M, Hofman M, Dvorsak M, Nicholson JK, Wilson ID (1992) *J Pharm Biomed Anal* **10**: 601–05.
6 Seddon MJ, Spraul M, Wilson ID, Nicholson JK, Lindon JC (1994) *J Pharm Biomed Anal* **12**: 419–24.
7 Spraul M, Hofman M, Lindon JC, Nicholson JK, Wilson ID. (1993) *Analytical Proceedings* **30**: 390–92.
8 Bäcker AE, Thorbert S, Olling A, Hallberg EC, Gustavsson M, Samuelsson BE, Soussi B (1995) *Glycoconj J,* **12**: 404.
9 Olling A, Sandberg P, Bäcker AE, Hallberg EC, Larson G, Samuelsson BE, Soussi B (1997) *J Magn Reson.* In press.
10 Karlsson K-A (1987) *Methods Enzymol* **138**: 212–20.
11 Holgersson J, Jovall P, Samuelsson BE, Breimer ME (1991) *Glycoconj J* **8**: 424–33.
12 Li S, De Gasperi R, Muldrey JE, Li Y-T (1986) *Biochem Biophys Res Commun* **141**: 346–52.
13 Hansson GC, Li Y, Karlsson H (1989) *Biochemistry* **28**: 6672–78.
14 Larson G, Karlsson H, Hansson GC, Pimlott W (1987) *Carbohydr Res* **161**: 281–90.
15 Magnani JL, Smith DF, Ginsburg V (1980) *Anal Biochem* **109**: 399–402.
16 Hansson GC, Karlsson K-A, Larson G, Samuelsson BE, Thurin J (1985) *J Immunol Methods* **83**: 37–42.
17 Hynsjö L, Granberg L, Haurum J, Thiel S, Larson G (1995) *Anal Biochem* **225**: 305–14.
18 Bouhours D, Liaigre J, Naulet J, Maume D, Bouhours JF (1997) *Glycoconj J* **14**: 29–38.
19 Hansson G, Karlsson H (1993) *Methods Mol Biol* **14**: 47–54.
20 Strecker G, Wieruszeski J-M, Michalski J-C, Montreuil J (1989) *Glycoconj J* **6**: 271–84.
21 Kannagi R, Levery SB, Ishigami F, Hakomori S, Shevinsky LH, Knowles BB, Solter D (1983) *J Biol Chem* **258**: 8934–42.
22 Domon B, Costello CE (1988) *Glycoconj J* **5**: 397–409.
23 Karlsson H, Carlstedt I Hansson GC (1989) *Anal Biochem* **182**: 438–46.
24 Egge H (1978) *Chem Phys Lipids* **21**: 349–60.
25 Bouhours D, Bouhours JF, Larson G, Karlsson H, Pimlott W, Hansson GC (1990) *Arch Biochem Biophys* **282**: 141–46.
26 Griffiths L (1995) *Anal Chem* **67**: 4091–95.
27 Spraul M, Hofman M, Lindon JC, Farrant RD, Seddon MJ, Nicholson JK, Wilson ID (1994) *NMR Biomed* **7**: 295–303.
28 Hallberg EC, Holgersson J, Samuelsson BE (1998) *Glycobiology.* **8**:637–49.
29 Holgersson J, Stömberg N, Breimer ME (1988) *Biochimie* **70**: 1565–74.
30 von dem Borne AE, Bos MJ, Joustra-Maas N, Tromp JF, van't Veer MB, van Wijngaarden-du Bois R, Tetteroo PA (1986) *Br J Haematol* **63**: 35–46.
31 Clausen H, Levery SB, Nudelman E, Baldwin M, Hakomori S (1986) *Biochemistry* **25**: 7075–85.
32 Clausen H, Levery SB, Nudelman E, Tsuchiya S, Hakomori S (1985) *Proc Natl Acad Sci USA* **82**: 1199–1203.
33 Good AH, Yau O, Lamontagne LR, Oriol R (1992) *Vox Sang* **62**: 180–89.
34 Springer T, Galfré G, Secher DS, Milstein C (1978) *Eur J Immunol* **8**: 539–51.
35 Abe K, Levery SB, Hakomori S (1984) *J Immunol* **132**: 1951–54.
36 Young WWJ, Portoukalian J, Hakomori S (1981) *J Biol Chem* **26**: 10967–72.
37 Watarai S, Handa S, Tadakuma T, Yasuda T (1987) *J Biochem* (Tokyo) **102**: 59–67.
38 Clausen H, Levery SB, McKibbin JM, Hakomori S (1985) *Biochemistry* **24**: 3578–86.
39 Holgersson J, Jovall P, Samuelsson BE, Breimer ME (1990) *J Biochem* (Tokyo) **108**: 766–77.
40 Koscielak J, Piasek A, Gorniak H, Gregor A (1973) *Eur J Biochem* **37**: 214–25.
41 Stellner K, Watanabe K, Hakomori S (1973) *Biochemistry* **12**: 656–61.
42 Lindström K, Breimer ME, Jovall PA, Lanne B, Pimlott W, Samuelsson BE (1992) *J Biochem* (Tokyo) **111**: 337–45.
43 Holgersson J, Curtis JM, Morris MR, Tetler LW, Derrick PJ, Samuelsson BE (1993) *Rapid Commun Mass Spectrom* **7**: 421–26.
44 Henry SM, Woodfield DG, Samuelsson BE, Oriol R (1993) *Vox Sang* **65**: 62–69.
45 Smith EL, McKibbin JM (1975) *J Biol Chem* **250**: 6059–64.
46 Hansson GC, Karlsson K-A, Larson G, McKibbin JM, Strömberg N, Thurin J (1983) *Biochim Biophys Acta* **750**: 214–16.
47 McKibbin JM (1978) *J Lipid Res* **19**: 131–47.
48 Hansson GC, Karlsson K-A, Leffler H, Strömberg N (1982) *FEBS Lett* **139**: 291–94.

Received 9 April 1998, revised 18 January 1999, accepted 4 February 1999