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SIMULTANEOUS DETERMINATION OF N-ACETYLGLUCOSAMINE, N-ACETYLGALACTOSAMINE, N-ACETYLGLUCOSAMINITOL AND N-ACETYLGALACTOSAMINITOL BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A gas–liquid chromatographic procedure is described which will concomitantly separate and quantitate N-acetylglucosamine, N-acetylgalactosamine, N-acetylglucosaminitol and N-acetylgalactosaminitol in a single analytical run. The hexosamines, as their O-methyloximes, and the hexosaminitols can be separated as either their per-O-acetylated or per-O-trimethylsilylated derivatives. This method is particularly useful with samples that possess both N-acetylhexosaminitols and N-acetylhexosamines as are seen with N-linked oligosaccharides that are cleaved from glycoproteins by alkaline borohydride treatment. This procedure demonstrates a range of acceptable linearity of 1–1000 nmoles for each type of amino sugar.

INTRODUCTION

Both N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) have been identified as being involved in the linkage region of side-chain oligosaccharides to the protein core of many glycoproteins[1]. Typically, GlcNAc is bound to asparagine (GlcNAc–Asp) in the formation of N-linked oligosaccharides, commonly found on plasma glycoproteins, and GalNAc is covalently bound to serine and threonine (GalNAc–Ser/Thr) forming the O-linked oligosaccharides, generally found on epithelial mucins[2]. In the analysis of glycoprotein side-chain oligosaccharides the GalNAc–Ser/Thr glycosidic linkage is usually cleaved under mild alkaline conditions in the presence of sodium borohydride[3,4]. The GlcNAc–Asp linkage is cleaved under stronger alkaline conditions in the presence of sodium borohydride[3,5]. In both cases the oligosaccharide side-chains are released from the protein core and the aldehyde of the hexosamine originally involved in the protein–oligosaccharide linkage is concomitantly reduced to the corresponding N-acetylglucosaminitol (GlcNAc-ol) or N-acetylgalactosaminitol (GalNAc-ol) sugar alcohol.

For monosaccharide analysis the released side-chained oligosaccharides are then subjected to acid hydrolysis and the resulting sugar monomers are generally reduced to their respective sugar alcohols with sodium borohydride. Following ace-

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tylation, the hexosaminitols and the neutral sugar alcohols are then concomitantly quantified by capillary[6,7] or packed-column[8] gas–liquid chromatography (GLC). Via this method all oligosaccharide N-hexosamine residues, *i.e.*, those involved in the protein–oligosaccharide linkage and those within the oligosaccharide structure, form the same respective hexosaminitol derivative and thus provide no structural information.

A sensitive and quantitative GLC–mass spectrometry (MS) method has been reported that is able to differentiate between the GalNAc involved in the N-glycosidic linkage of an oligosaccharide to the protein core and other GalNAc residues that are not involved in this linkage but are present within the oligosaccharide structure[9]. By this procedure a glycoprotein possessing an oligosaccharide with GalNAc as a linkage sugar is treated with alkaline sodium borohydride which effectively cleaves the oligosaccharide from the protein core and reduces the GalNAc linkage sugar to GalNAc-ol. The sample is then hydrolyzed and the resulting sugars are reduced with sodium borodeuteride and then acetylated. By substituting sodium borodeuteride in the latter reduction step all but the previously reduced linkage sugar (*i.e.*, GalNAc-ol) form derivatives that possess deuterium on carbon-1. GLC–MS is then used to determine the ratio of deuterium-labeled and hydrogen-labeled carbon-1 containing ion pairs which gives the ratio of GalNAc to GalNAc-ol.

An alternative to this method employs the amino acid analyzer to separate the hydrochloride salts of the hexosaminitols and hexosamines that are produced as the result of acid hydrolysis of alkaline borohydride released oligosaccharides[10]. Though this method provides excellent results, it requires a two-buffer system, modification of the ninhydrin reaction coil and approximately 2 h per run-time analysis.

To date, no capillary or packed-column GLC procedure is available which allows for the simultaneous separation and quantitation of GlcNAc, GalNAc, GlcNAc-ol and GalNAc-ol. The present investigation reports a GLC method that enables in a single analysis the separation and quantification of both the individual hexosamines involved and not involved in the oligosaccharide linkage region. Following the cleavage and reduction of the glycoprotein oligosaccharide side-chains and subsequent hydrolysis and re-N-acetylation of the hexosamines, the non-reduced hexosamines are derivatized to their respective O-methyloxime acetates and the existing hexosaminitols, which do not react to form the oxime, form their respective hexosaminitol acetates. Each of these acetylated derivatives is then separated on a polar GLC column. Alternatively, the resulting O-methyloxime and hexosaminitol mixture can be trimethylsilylated and then quantitated by GLC. Because of this flexibility in the choice of hexosamine derivative and of GLC column it is possible, in many cases, to perform the concomitant analysis of neutral carbohydrates as well.

MATERIALS AND METHODS

Instrumentation

Gas chromatography was performed using a Perkin-Elmer (Norwalk, CT, U.S.A.) Sigma 3 instrument equipped with dual flame ionization detectors. Chromatographic columns were 6 ft. × 1/8 in. O.D. (1.8 mm I.D.), glass columns packed with either 3.0% diethylene glycol adipate (DEGA), stabilized (Foxboro/Analabs, North Haven, CT, U.S.A.), on Chromosorb W HP, 100–120 mesh, with 3% SP-2250

(Supelco, Bellefonte, PA, U.S.A.) on Supelcoport, 100–120 mesh or with 3% SP-2340 on Supelcoport, 100–120 mesh. Peak areas were measured using a Shimadzu (Rockville, MD, U.S.A.) C-R3A Chromatopac integrator.

Materials

All carbohydrate standards were obtained from Pfanstiehl Laboratories (Waukegan, IL, U.S.A.). Pyridine, methanol, 1-dimethylamino-2-propanol, acetic anhydride, hydroxylamine hydrochloride and sodium borohydride were purchased from Aldrich (Milwaukee, WI, U.S.A.). All solvents were redistilled prior to use. Sodium borodeuteride was obtained from Alfa (Danvers, MA, U.S.A.). O-Methylhydroxylamine hydrochloride was obtained from Pierce (Rockford, IL, U.S.A.).

Isolation of glycoproteins

All glycoproteins and glycopeptides were purified as described in the literature cited below. For consistency in the isolation methods, the oligosaccharides from each glycoconjugate were cleaved from the protein core via the appropriate alkaline-borohydride procedure described below. Subsequent purification of the isolated oligosaccharides was performed as described in each respective study. Oligosaccharide IV and an acidic pentasaccharide from porcine submaxillary mucin were isolated by the procedure of Carlson[11] and by Aminoff *et al.*[12], respectively. The glycopeptide I oligosaccharide from human serum thyroxine-binding globulin was prepared as described by Zinn *et al.*[13] and the desialated oligosaccharide from bovine prothrombin was isolated by the procedure of Mizuochi *et al.*[14]. Tracheobronchial mucus glycoprotein (TBG) from sputum from a patient with cystic fibrosis was prepared as outlined by Boat *et al.*[15] without carboxymethylation of cysteine residues. Isolation of the 3a(1) neutral pentasaccharide from TBG was performed as described by Van Hanlbeek *et al.*[16].

Reductive cleavage of the oligosaccharide–protein linkage

Mild alkaline-borohydride cleavage of the base labile GalNAc–Ser/Thr linkages via beta elimination was performed by treating each glycoprotein or glycopeptide (50–500 μg) with 0.05 *M* sodium hydroxide–1.0 *M* sodium borohydride (1.0 ml/mg sample) at 45°C for 15 h. Cleavage of the base resistant GlcNAc–Asp linkage via alkaline hydrolysis was accomplished by dissolving the glycoprotein or glycopeptide (50–500 μg) in 1.0 *M* sodium hydroxide–1.0 *M* sodium borohydride (1.0 ml/mg sample) and heating it at 100°C for 6 h. D-Glucoheptitol was added as internal standard. At the end of the respective incubation period dilute acetic acid was added to acidify each solution which was then passed through a 3 cm × 0.8 cm I.D. column of Dowex 50W-X8 (hydrogen form, 50–100 mesh). Each sample was then dried to a syrup *in vacuo* and the boric acid removed by the sequential addition and evaporation of 5 × 1.0 ml dry methanol.

Hydrolysis of oligosaccharides

Oligosaccharides and methyl glycoside standards were each subjected to acid hydrolysis in 0.2 ml 4.0 *M* hydrochloric acid at 100°C for 4 h under nitrogen atmosphere. Samples were then dried *in vacuo* at 30°C using a rotary evaporator, redissolved in 0.25 ml of 95% ethanol to which was then added 2.0 ml benzene, and were again dried. This latter step was repeated twice.

Derivatization of carbohydrates

Hydrolyzed samples to be oximated and trimethylsilylated, or to be reduced with sodium borohydride or sodium borodeuteride to the alditols and acetylated, were first treated with pyridine and acetic anhydride in dry methanol by the procedure of Kozulic *et al.*[17] to re-N-acetylate the hexosamines. The per-O-acetyl O-methyloxime, the per-O-trimethylsilyl O-methyloxime and the per-O-trimethylsilyl O-trimethylsilyloxime derivatives of the hexosamines and neutral sugars, and the aldononitrile acetates of the neutral sugars were prepared as previously described[18].

Samples to be O-methyloximated and acetylated were treated in two ways. In the first way, a sample was divided into two equal fractions with one being O-methyloximated and acetylated for hexosamine analysis and the other analyzed for their content of neutral sugars as their alditol acetates[6–8] or aldononitrile acetates[18]. Alternatively, the hexosamines and hexosaminitols in a sample were separated from the neutral sugars and isolated with the use of cation-exchange resins by the procedure of Boas[19]. The hexosamines and hexosaminitols were then subjected to O-methyloximation and acetylation and the neutral sugars analyzed as alditol acetates or aldononitrile acetates.

For comparison to other analytical methods each oligosaccharide was also analyzed for amino sugars by GLC–MS and by analysis on an amino acid analyzer as described by Weber and Carlson[9] and Cheng and Boat[10], respectively.

RESULTS AND DISCUSSION

GLC separations

The GLC separation of both the per-O-acetylated O-methyloxime and per-O-acetylated alditol derivatives of N-acetylglucosamine and N-acetylgalactosamine on a 3% DEGA, stabilized, column is presented in Fig. 1. As shown, each amino sugar derivative displayed a single symmetrical chromatographic peak and exhibited no significant peak tailing. Though it has been shown that when a sugar is oximated, two isomeric products are produced, representing the *syn* and *anti* forms of the O-methyloxime function[20], these were unresolved for the per-O-acetylated O-methyloximated N-acetylhexosamines on the DEGA column[18].

It should be noted that the per-O-acetylated O-methyloxime derivatives of neutral pentoses and hexoses emerge from the GC column well before the hexosamines and do not interfere with their analysis. Though this is of little use for the analysis of the neutral sugars as these derivatives on the DEGA column, since they exhibit overlapping *syn* and *anti* peaks, it does allow for the analysis of the amino sugars even when neutral sugars are present in the sample. Because of this, a sample containing both amino and neutral sugars can simply be divided into two aliquots of which one is analyzed for amino sugars, as described above. The per-O-acetylated O-methyloximes of the neutral sugars that are present in this aliquot elute early from the column and are not assayed. The neutral sugars in the second aliquot can then be separately quantitated as their aldononitrile acetates[18], alditol acetates[6–8] or by another method. In this way, the same internal standard (*e.g.*, myo-inositol, D-glucoheptitol, etc.) can be utilized in both analyses. Alternatively, especially for samples with low carbohydrate content, the amino sugars can be separated from the neutral sugars by ion-exchange chromatography[19] and then each can be analyzed separately.

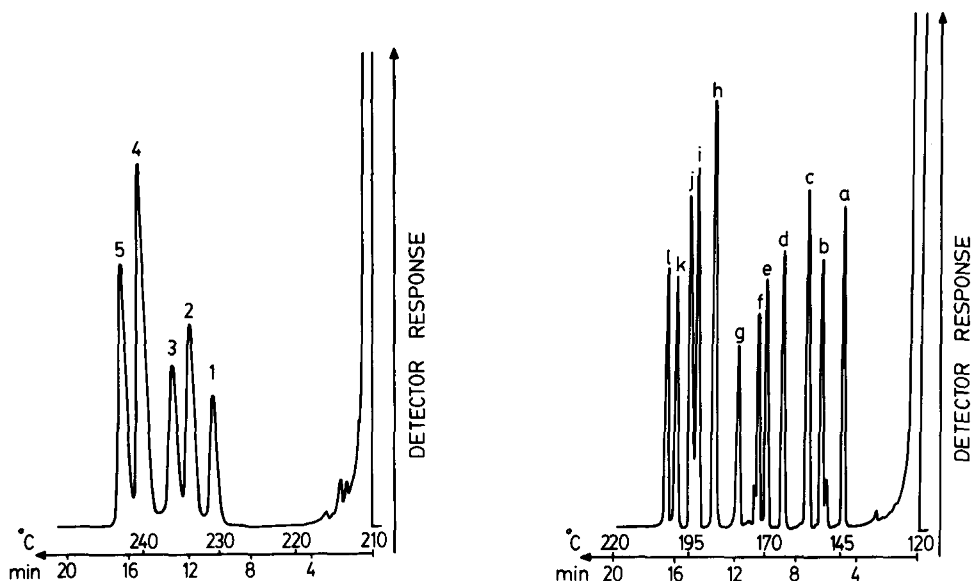

Fig. 1. Gas–liquid chromatogram of the per-O-acetylated derivatives of (1), D-glucoheptitol = internal standard; (2), (1-O-methyloxime)-N-acetylglucosamine; (3), (1-O-methyloxime)-N-acetylgalactosamine; (4), N-acetylglucosaminitol and (5), N-acetylgalactosaminitol. Injected sample contained 0.5 μg of each oximated amino sugar and internal standard and 1 μg of each hexosaminitol. Sample was separated on a 3.0% DEGA (stabilized) on Chromosorb W HP, 100–120 mesh, GC column. Program: temperature was increased from 210°C to 240°C at a rate of 2°C/min. Nitrogen was used as the carrier gas at a flow-rate of 20 ml/min.

Fig. 2. Gas–liquid chromatogram of the per-O-trimethylsilyl derivatives of (a), (1-O-methyloxime)-2-deoxy-D-ribose; (b), (1-O-methyloxime)-D-ribose; (c), (1-O-methyloxime)-L-fucose; (d), (1-O-methyloxime)-2-deoxy-D-glucose; (e), (2-O-methylketoxime)-D-fructose; (f), (1-O-methyloxime)-D-glucose; (g), (1-O-methyloxime)-D-glucuronic acid; (h), myo-inositol = internal standard; (i), N-acetylglucosaminitol; (j), N-acetylgalactosaminitol; (k), (1-O-methyloxime)-N-acetylglucosamine and (l), (1-O-methyloxime)-N-acetylgalactosamine. Unlabeled small peaks at the base of (b) D-ribose and (f) D-glucose represent the resolved *syn* or *anti* isomer of that respective sugar. Injected sample contained 1 μg of each oximated amino sugar, internal standard and hexosaminitol. Sample was separated on a 3.0% SP-2250 Supelcoport, 100–120 mesh, GC column. Program: temperature was increased from 120°C to 220°C at a rate of 5°C/min. Nitrogen was used as the carrier gas at a flow-rate of 18 ml/min.

Fig. 2 shows the separation of the trimethylsilyl derivatives of GlcNAc-ol and GalNAc-ol and of the per-O-trimethylsilyl O-methyloximes of GlcNAc, GalNAc and several neutral sugars on a 3% SP-2250 GLC column. Although the major peaks in this chromatogram may be easily integrated, baseline separation of the N-acetylated hexosaminitols is not achieved as it is for the O-methyloximated amino sugars. The *syn* and *anti* forms of the O-methyloxime function[18] were unresolved for the amino sugars and most neutral sugars[20] as is evident by their single symmetrical GC peaks. Ribose and glucose, on the other hand, each demonstrate a large peak with a small side peak which is indicative of resolved *syn* and *anti* isomers[20]. For glycoproteins containing N-linked oligosaccharides which possess only one type of hexose, though, this method is particularly useful since the reducible hexosamines and neutral sugars in the sample are both derivatized to their respective O-methyloximes and thus do

TABLE I

GAS CHROMATOGRAPHIC RETENTION DATA FOR ACETYLATED AND FOR TRIMETHYLSILYLATED DERIVATIVES OF 2-ACETAMIDO-2-DEOXY-HEXOSAMINES

Retention times given in min. Retention times presented in parentheses indicate the presence of a second peak due to separation of *syn* and *anti* isomers of the oxime.

	*DEGA**	*DEGA***	*SP-2340****	*SP-2250*§
O-methyloxime acetates				
N-Acetylglucosamine	11.95	8.40	8.85 (8.91)	–
N-Acetylgalactosamine	13.02	9.52	10.80 (10.93)	–
N-Acetylmannosamine	13.56	9.68	10.85	–
Alditol acetates				
N-Acetylglucosaminitol	15.15	11.90	17.12	–
N-Acetylgalactosaminitol	16.25	12.60	19.83	–
N-Acetylmannosaminitol	16.32	12.71	19.87	–
O-Methyloxime trimethylsilyl ethers				
N-Acetylglucosamine	–	–	–	16.20
N-Acetylgalactosamine	–	–	–	16.75
N-Acetylmannosamine	–	–	–	16.79
Alditol trimethylsilyl ethers				
N-Acetylglucosaminitol	–	–	–	14.60
N-Acetylgalactosaminitol	–	–	–	15.25
N-Acetylmannosaminitol	–	–	–	15.28

* 3% DEGA, stabilized, on Chromosorb W HP, 100–120 mesh. Column: glass, 6 ft. × 1/8 in. O.D. (1.8 mm I.D.). Program: temperature was increased from 210°C to 240°C at a rate of 2°C per/min. Nitrogen was used as the carrier gas at a flow-rate of 20 ml/min.

** 3% DEGA, stabilized, on Chromosorb W HP, 100–120 mesh. Column: glass, 6 ft. × 1/8 in. O.D. (1.8 mm I.D.). Program: isothermal at 230°C. Nitrogen was used as the carrier gas at a flow-rate of 20 ml/min.

*** 3% SP-2340 on Supelcoport, 100–120 mesh. Column: glass, 6 ft. × 1/8 in. O.D. (1.8 mm I.D.). Program: isothermal at 240°C. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

§ 3% SP-2250 on Supelcoport, 100–120 mesh. Column: glass, 6 ft. × 1/8 in. O.D. (1.8 mm I.D.). Program: temperature was increased from 120°C to 260°C at a rate of 5°C per/min. Nitrogen was used as the carrier gas at a flow-rate of 18 ml/min.

not require prior separation of hexosamines from the neutral sugars before derivatization. In contrast to the per-O-trimethylsilyl O-methyloximes of GlcNAc and GalNAc (Fig. 2), no separation of GlcNAc and GalNAc could be achieved as their per-O-trimethylsilyl O-trimethylsilyloximes (not shown) and, in addition, the per-O-trimethylsilyl O-trimethylsilyloximes of any hexoses that may be in a sample interfere with the trimethylsilylated hexosaminitol peaks. Lastly, it should be noted that trimethylsilylation of oximated hexosamines in their hydrochloride salt or free base form produced derivatives that were both degraded readily on the GLC column and are not useful in resolving GlcNAc from GalNAc and GlcNAc-ol from GalNAc-ol. Re-N-acetylation of the free amines prior to trimethylsilylation[17] prevented their degradation on the GLC column and resulted in the separation of the different hexosamine derivatives, as described above.

Retention data

Retention data for each acetylated derivative on 3% DEGA and 3% SP-2340 and each trimethylsilyl derivative on 3% SP-2250 are given in Table I. On the polar DEGA and the very polar SP-2340 columns the hexosaminitol acetates elute from the column after the O-methyloxime acetates. This elution order is reversed when the trimethylsilyl derivatives of these amino sugars are separated on an intermediate polar 3% SP-2250 GC column. As indicated above, no separation of *syn* and *anti* isomers of the O-methyloximes of the N-acetylhexosamines was observed on the DEGA column but was evident as closely related peaks on SP-2340. Under the same chromatographic conditions, retention times for all hexosamine derivatives were typically shorter on the DEGA column than on the SP-2340 column. Notably, the SP-2340 packed column demonstrated significant column bleed at the higher operating temperatures required to separate the amino sugars. Lastly, isothermal separation of the hexosamine derivatives can also be performed on the 3% DEGA column which readily permits sample injections every 20 min. This run time can be reduced further by utilizing a 1.5% DEGA column (not shown).

Quantitative aspects

Employing N-acetylhexosamine and N-acetylhexosaminitol standards a linear response curve in the range of 1 to 1000 nmoles was obtained for each sugar using flame ionization as the GLC detector. In general practice, though, for the analysis of hydrolyzed biological samples a working range of 2 to 100 nmoles for amino sugars was typically employed.

Analysis of purified oligosaccharides

Since excellent separation of the amino sugar alcohols and the O-methyloximated amino sugars is achieved as their per-O-acetylated derivatives on a 3% DEGA column (Fig. 1) this procedure is readily applicable to samples possessing both types of amino sugars, such as hydrolyzed N-linked oligosaccharides that were liberated from glycoproteins via alkaline borohydride treatment. In the latter case, the amino sugar involved in the N-linkage to the protein core would have been reduced to its respective N-acetylhexosaminitol upon cleavage and, following acid hydrolysis of the liberated oligosaccharide, the amino sugars that were present within the oligosaccharide structure would be reducible and would form O-methyloximes whereas the N-acetylhexosaminitol would not. Table II presents the results of the N-acetylhexosamine analysis of representative oligosaccharides that were cleaved from different glycoproteins by alkaline borohydride treatment. The neutral sugars in these samples were assayed as their aldononitrile acetates[18]. Five separate samples (10 μg each) of each alkaline borohydride released oligosaccharide were subjected to acid hydrolysis. Following the O-methyloximation of the reducible amino sugars with O-methylhydroxylamine the O-methyloximated amino sugars and the hexosaminitols in each sample were analyzed as their per-O-acetylated derivatives on a 3% DEGA column, as described above. Three of the oligosaccharides were reported to have GalNAc involved in the original protein–oligosaccharide linkage and the other two involved GlcNAc in the linkage. In addition, these specific oligosaccharides were chosen because they also possess amino sugars other than the linkage hexosamine within their structure. As shown in Table II, analysis of the amino sugars, via this method (GLC),

TABLE II

N-ACETYLHEXOSAMINE ANALYSIS OF SOME KNOWN ALKALINE BOROHYDRIDE RELEASED GLYCOPROTEIN OLIGOSACCHARIDES

Data expressed as the mean weight (in μg) with the standard deviation in parentheses of five separate analyses and the mole ratio in brackets of each carbohydrate relative to the identified N-acetylhexosaminitol (involved in the protein–oligosaccharide linkage) found in each sample. In the GLC method, amino sugars were assayed as described in Fig. 1 and their neutral sugars were determined as their aldononitrile acetates[18]. For comparison the same oligosaccharides were assayed for amino sugars by a GLC–MS method[9] in which GlcNAc-ol and GalNAc-ol (protein-linked amino sugar), and GlcNAc and GalNAc (non-protein-linked amino sugars) were determined by the mass ratio of hydrogen-labeled and deuterium-labeled C-1 fragments, respectively. The neutral sugars in these samples were assayed as their alditol acetates[8]. Abbreviations: GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; GlcNAc-ol, N-acetylglucosaminitol; GalNAc-ol, N-acetylgalactosaminitol; Fuc, D-fucose; Man, D-mannose; Gal, D-galactose.

Oligosaccharide	*Reference*	*Method*
Fuc–Gal–GalNAc-ol | GalNAc	11	GLC GLC–MS
NeuNGl–GalNAc-ol | GalNAc–Gal | Fuc	12	GLC GLC–MS
NeuNAc–Gal–GlcNAc–Man \\ Man–GlcNAc–GlcNAc-ol / NeuNAc–Gal–GlcNAc–Man	13	GLC GLC–MS
Gal–GlcNAc–Man–Man–GlcNAc–GlcNAc–ol | Gal–GlcNAc–Man	14	GLC GLC–MS
Gal–GlcNAc–Gal–GalNAc-ol | Fuc	15, 16	GLC GLC–MS

concomitantly quantified both the amino sugar involved and not involved in the linkage to the protein core for each oligosaccharide structure. The small standard deviation observed for each series of amino sugar determinations clearly shows the excellent precision of analysis for this method. In all cases the results of the present method were in agreement with published structural data. Furthermore, the results were comparable with those amino sugar data acquired from the analysis of each of the above oligosaccharides by an alternate method (GLC–MS). This procedure is similar to the above method in that the oligosaccharides are cleaved from the protein core of the glycoproteins with alkaline borohydride but differs in that the reducible amino sugars (*i.e.*, those not involved in the protein linkage) that are formed following hydrolysis of the oligosaccharides are reduced to their corresponding hexosaminitols with sodium borodeuteride in lieu of oximation. GLC is then used to separate the per-O-acetylated hexosaminitol derivatives of N-acetylglucosamine and N-acetylgalactosamine. Analysis of each hexosaminitol GLC peak by MS provides

GlcNAc	*GalNAc*	*GlcNAc-ol*	*GalNAc-ol*	*Fuc*	*Man*	*Gal*
–	4.21(0.02) [1.04]	–	4.08(0.04) [1.00]	2.88(0.13) [0.96]	–	3.06(0.12) [0.93]
–	3.66(0.13) [0.90]	–	4.11(0.12) [1.00]	2.84(0.04) [1.02]	–	3.11(0.03) [1.02]
–	3.31(0.02) [0.97]	–	3.44(0.04) [1.00]	2.63(0.13) [1.04]	–	2.80(0.12) [1.01]
–	2.98(0.15) [0.89]	–	3.38(0.16) [1.00]	2.38(0.05) [0.96]	–	2.67(0.03) [0.98]
3.66(0.03) [2.93]	–	1.26(0.02) [1.00]	–	–	3.16(0.11) [3.11]	1.99(0.10) [1.96]
3.48(0.16) [2.85]	–	1.20(0.13) [1.00]	–	–	2.92(0.04) [3.02]	1.99(0.03) [1.99]
5.08(0.01) [3.05]	–	1.68(0.02) [1.00]	–	–	4.15(0.12) [3.06]	2.67(0.11) [1.97]
5.01(0.16) [2.89]	–	1.75(0.11) [1.00]	–	–	4.15(0.04) [2.94]	2.79(0.05) [1.98]
3.14(0.03) [0.97]	–	–	3.27(0.03) [1.00]	2.26(0.12) [0.94]	–	5.58(0.12) [1.98]
2.88(0.12) [0.93]	–	–	3.10(0.14) [1.00]	2.19(0.04) [0.96]	–	5.02(0.05) [2.01]

the ratio of amino sugar reduced with sodium borohydride and reduced with sodium borodeuteride by measuring the ratio of hydrogen-labeled and deuterium-labeled C-1 containing fragment ion pairs, representing the protein-linked and non-protein-linked amino sugars, respectively. The neutral sugars in these samples were assayed as their alditol acetates[8].

Fig. 3 illustrates the GC separation of the amino sugars found in the porcine blood group H substance oligosaccharide fraction 14.5[21] employing D-glucoheptitol as the internal standard. As shown, excellent separation of the amino sugars N-acetylglucosamine and N-acetylgalactosaminitol is achieved on the 3% DEGA, stabilized, GLC column. Also shown in Fig. 3 is the concomitant separation of the oligosaccharide's neutral sugars as their aldononitrile acetates with β-methyl-D-glucopyranoside as the internal standard. This was accomplished by separating the amino sugars from the neutral sugars after hydrolysis of the oligosaccharide. Following the addition of the respective internal standard the amino sugar fraction was

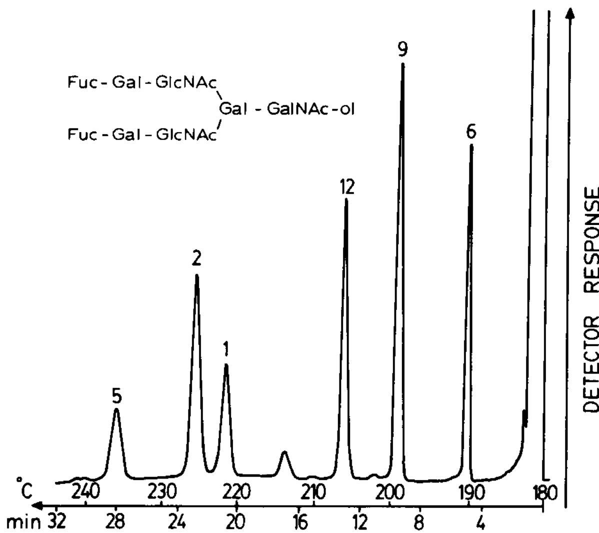

Fig. 3. GC separation of the amino sugars and neutral sugars (aldononitrile acetates) of the porcine blood group H substance oligosaccharide fraction 14.5[18] on a 3% DEGA GLC column. Following hydrolysis of the oligosaccharide (8.2 μg), the sample's hexosamines were separated from the neutral sugars. After the addition of the respective internal standards and subsequent derivatizations the sugars were then recombined for GLC analysis. (1) D-glucoheptitol = internal standard for amino sugars; (2) (1-O-methyloxime)-N-acetylglucosamine; (5) N-acetylgalactosaminitol; (9) β-methyl-D-glucopyranoside = internal standard for neutral sugars, and the aldononitrile acetates of (6) L-fucose and (12) D-galactose. The unassigned peak at approximately 17 min is a side-product cyclic oxime produced during the derivatization of L-fucose to its aldononitrile acetate. Sample was separated on a 3.0% DEGA (stabilized) on Chromosorb W HP, 100–120 mesh, GC column. Program: temperature was increased from 180°C to 240°C at a rate of 2°C/min. Nitrogen was used as the carrier gas at a flow-rate of 20 ml/min.

O-methyloximated and acetylated and the neutral sugars were derivatized to their aldononitrile acetates[18]. Both derivatized samples were recombined prior to GLC injection. The separated amino sugars and neutral sugars were then quantitated against their own respective internal standard, thus eliminating errors in mixing. The

TABLE III

N-ACETYLHEXOSAMINE ANALYSIS OF THE PORCINE BLOOD GROUP H SUBSTANCE OLIGOSACCHARIDE FRACTION 14.5

Data expressed as the mean weight (in μg) with the standard deviation in parentheses of five separate analyses and the mole ratio in brackets of each carbohydrate relative to N-acetylgalactosaminitol in the sample. Amino sugars were assayed as described in Fig. 1 and their neutral sugars were determined as their aldononitrile acetates[15]

Oligosaccharide	*Reference*	*GlcNAc*	*GalNAc-ol*	*Fuc*	*Gal*
Fuc–Gal–GlcNAc \ Gal–GalNAc-ol / Fuc–Gal–GlcNAc	15	3.66(0.03) [2.93]	1.26(0.04) [1.00]	3.16(0.11) [3.11]	1.99(0.10) [1.96]

results of the analyses are presented in Table III. The unassigned peak at approximately 17 min (Fig. 3) is a by-product cyclic oxime of L-fucose[22]. Though shown being separated as their per-O-acetylated O-methyloxime and hexosaminitol derivatives for the amino sugars and as the aldononitrile acetates for the neutral sugars, and because of the few types of carbohydrates involved in this oligosaccharide, analysis could also have been performed as the per-O-trimethylsilyl O-methyloxime derivatives of both amino and neutral sugars, as mentioned above as being useful for oligosaccharides which possess amino sugars and only one type of hexose. Since the sample's reducible hexosamines and neutral sugars are both derivatized to, and analyzed as, their respective per O-trimethylsilyl O-methyloxime derivatives, no prior separation of hexosamines from the neutral sugars before derivatization is needed.

CONCLUSION

Two GLC methods are described which permit the simultaneous determination of GlcNAc, GalNAc, GlcNAc-ol and GalNAc-ol. Both procedures are based upon the O-methyloximation of GlcNAc and GalNAc but differ in that the resulting O-methyloximated N-acetylhexosamines along with the N-acetylhexosaminitols are then per-O-acetylated or per-O-trimethylsilylated. These methods are particularly useful with samples that possess both N-acetylhexosaminitols and N-acetylhexosamines as are seen with N-linked oligosaccharides cleaved from glycoproteins by alkaline borohydride treatment. Though the examples given were focused on purified oligosaccharides which possessed only one type of N-acetylhexosaminitol (*i.e.*, the protein-linked amino sugar), these methods are readily applicable to alkaline borohydride cleaved mixtures of oligosaccharides which may contain both GlcNAc-ol and GalNAc-ol. This would provide valuable quantitative information on the number and types of N-linked oligosaccharides present in the sample. Furthermore, since these methods concomitantly measure GlcNAc and GalNAc they would also simultaneously provide quantitative data on the N-acetylhexosamines that are present within the oligosaccharides and not involved in the N-linkage to the protein core of the glycoprotein.

Lastly, with regards to the analysis of the neutral sugars in these samples, there is considerable flexibility with the application of the above amino sugar methods. With small samples, analysis of the amino sugars via their per-O-acetylated O-methyloxime and per-O-acetylated alditol derivatives on a 3% DEGA GLC column (Fig. 1) is best accomplished by the separation of the amino sugars from the neutral sugars before derivatization with the latter being analyzed separately as their aldononitrile acetates, alditol acetates or by another method. Provided there is no interference from the by-product cyclic oximes of the neutral sugar aldononitrile acetates (as seen with xylose and arabinose) it is possible to recombine these derivatives with the derivatized amino sugars and analyze them together on the 3% DEGA GLC column, quantitating them against their own respective internal standard. With larger samples, and employing the same per-O-acetylated derivatives, it is not necessary to separate the amino sugars from the neutral samples for quantitation but rather the sample can be simply divided into two aliquots with one being assayed directly for amino sugars (as their O-methyloximes) and the other for neutral sugars (by another GLC method), as described above. Alternatively, the reducing carbohydrates in a

single sample can be O-methyloximated and then all the sugars per O-trimethylsilylated. GlcNAc, GalNAc, GlcNAc-ol, GalNAc-ol and the neutral sugars are then analyzed on a 3% SP-2250 GLC column with a single injection. As noted above, if more than one hexose is present an aliquot of the sample should be analyzed separately for neutral sugars by another method. Notably, since trimethylsilylated sugars are subject to eventual hydrolysis the samples should be analyzed shortly after derivatization.

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