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Structure–retention index relationships for derivatized monosaccharides on non-polar gas chromatography columns

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ABSTRACT

A gas chromatographic method for predicting the retention index of a derivatized monosaccharide is presented. The procedures are especially useful to detect and predict minute quantities of sugars in biological or chemical samples. Monosaccharides are first converted to the alditols and then derivatized by acetylation, permethylation or silylation. The derivatized monosaccharide structure–retention index relationship that has been developed is useful in the identification of unknown monosaccharides that can be readily confirmed by gas chromatography–mass spectrometry.

INTRODUCTION

In our studies of the post-translational modification of proteins [1,2] it was necessary to develop methods to identify minute amounts of unknown oligosaccharides from a glycoprotein. The characterization of the extremely polar, non-volatile, water-soluble saccharides of unknown structure from biological samples represents a formidable challenge to biochemists and chemists. Recently, however, methods to separate oligosaccharides or monosaccharides arising from hydrolysis of oligosaccharides [3] or glycoproteins [4] in complex biological or chemical mixtures has advanced to the point where this does not represent an obstacle to separating glycans in a mixture. Even though new high-pH anion-exchange chromatography coupled with pulsed amperometric detection has been useful for correlating retention times of neutral and acidic oligosaccharides with putative structures [5], methods for definitive detection and characterization of structures of saccharides are needed. Although a number of methods for separation and detection of saccharides are available including paper chromatography [6], thin-layer chromatography [7] and high-performance liquid chromatography [8], gas chromatography [9] and gas chromatography–mass spectrometry [10] are probably the methods of choice. For derivatized saccharides that can be separated by gas chromatography, this is an extremely sensitive method of detection. To date, however, it has not been particularly useful for unknown derivatized monosaccharides because currently no monosaccharide chemical structure–retention index relationship is available in the literature. Extensive studies of the relationship between retention index (I) and chemical structure of many classes of compounds including esters [11], substituted benzenes [12], substituted [13,14] and non-substituted polycyclic aromatic hydrocarbons [15,16], amines [17], alcohols [17] and carboxylic acids [17] have been reported in the literature. It has been shown that tentative identification of organic compounds can be made from the I values on non-polar gas chromatography columns using a method where the unknown peak is compared with known standards in order to predict the structure or propose a probable structure for a compound.

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In this report we present a gas chromatography method for the prediction of the chemical structure of a monosaccharide based on the I value from monosaccharide chemical structure–retention index relationships of known sugars. The advantages of this method are (a) determination of putative monosaccharide structures in the low pmol level, (b) the straightforward and short derivatization and run times, (c) the lack of requirement for radioactive or difficult-to-handle materials and (d) the excellent sensitivity and separation of very similar monosaccharides.

EXPERIMENTAL

Materials

Monosaccharides were obtained from Sigma (St. Louis, MO, USA). Acetic anhydride was obtained from Supelco (Bellefonte, PA, USA). Sodium hydroxide, sodium borohydride, sodium borodeuteride, anhydrous hexane, and perchloric acid were obtained from Aldrich (Milwaukee, WI, USA). N,O-Bis(trimethylsilyl) acetamide (BSA), trimethylchlorosilane (TMCS), water and acetic acid were obtained from Pierce (Rockford, IL, USA). All other chemicals and reagents were obtained in the highest quality available from commercial sources.

Instrumental analysis

Gas chromatography

All chromatographic runs were carried out on a Hewlett-Packard Model 5890 gas chromatograph, equipped with a flame ionization detector. A fused DB-1 capillary column (30 m × 0.322 mm I.D., film thickness 1 μm) was used. Helium was used as the carrier gas at a flow-rate of 60 ml/min. The injector and detector temperatures were maintained at 300°C. Two temperature programs were used: (A) 100°C for 3 min, followed by a 8°C/min rise to 200°C and hold for 1 min, to 280°C at 5°C/min and hold for 30 min; (B) 200°C for 4 min, followed by a 5°C/min rise to 280°C and hold for 30 min. Temperature program A was used for analysis of permethylated alditols and temperature program B was used for alditol acetates, and silylated sugars and alditols.

A reference mixture of standard n-alkanes (C_6–C_{36}) was injected simultaneously as a marker with each derivatized monosaccharide sample to be analyzed. Peak areas and retention time values were recorded using a Hewlett-Packard Model 3394 integrator. The I values were calculated using the equation of Van den Dool and Kratz [18]

$$I = 100\ i \cdot \frac{X - M_{(n)}}{M_{(n+i)} - M_{(n)}} + 100\ n$$

where n is the number of carbon atoms in the n-alkane marker, X, $M_{(n)}$, and $M_{(n+i)}$ are the adjusted retention times of the analyte, the normal alkane marker with n carbon atoms eluting before, and the alkane with $(n + i)$ carbon atoms eluting after the analyte, respectively; i is the interval and usually has the value of 1 or 2.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectra were obtained with a VG70S spectrometer fitted with a Varian Model 3600 gas chromatograph and a DB-1 capillary column (30 m × 0.25 mm I.D.). The carrier gas was helium. The linear temperature program was started at 80°C and increased to 300°C at 4°C/min. For chemical ionization mass spectrometry, ammonia was used as the reactant gas.

Chemistry

Reduction of the monosaccharide

The general procedure followed the methods previously described [19,20]. To the monosaccharide (100 μg), dried *in vacuo* by a speed-vacuo apparatus in a 0.3-ml Reacti-Vial (Pierce), a freshly prepared solution of sodium borohydride or sodium borodeuteride (100 μl of a 0.5 M solution) was added and allowed to stand overnight at room temperature. At the end of the reaction, acetic acid (60 μl of glacial acetic acid) was added and the entire mixture was dried *in vacuo*. At this point, the alditols resulting from the reduction of the monosaccharides were ready for derivatization.

Acetylation of alditols

The general procedure used was a modification of a method previously reported [21]. To the dried alditol, acetic anhydride (50 μl, 529 μmol), acetic acid (5 μl, 87 μmol) and perchloric acid (5 μl, 88 μmol of 60% aqueous perchloric acid) were introduced into the reaction vial. The mixture was sonicated for 2 min and allowed to stand for 8 min at room

temperature. At this point, water (200 μl, 11 mmol) was added followed by vigorous mixing. To the mixture was added dichloromethane (100 μl) and the mixture was vigorously mixed and the organic layer was separated by centrifugation. After separation, the aqueous phase was removed and the remaining dichloromethane fraction was washed with water (3 washes of 200 μl each). The alditol acetate was dried, dichloromethane (100 μl) was added and a sample (1 μl) of the acetylated alditol was directly injected onto the gas chromatograph or the gas chromatograph–mass spectrometer.

Permethylation of alditols

The general procedure used was based on previously published reports [22,23]. To the alditol or sugar, dimethylsulfoxide (300 μl) saturated with NaOH powder was added, followed by methyliodide (100 μl, 1.6 mmol). The mixture was vigorously mixed for 10 min. To the mixture was added water (1.0 ml), then dichloromethane (1.0 ml), and the mixture was vigorously mixed. Then the organic layer was separated from the aqueous layer by centrifugation. The aqueous fraction was removed and the remaining organic fraction was washed with water (3 washes of 3 ml each) and the organic fraction was dried with sodium sulphate, filtered, evaporated and taken up in dichloromethane (100 μl). A sample (1 μl) of the permethylated alditols was then directly injected into the gas chromatograph or the gas chromatograph–mass spectrometer.

Silylation of alditols

The general procedure for silylation of alditols was based on a previous report [24]. To the dried alditols or sugars, BSA (50 μl, 204 μmol) and TMCS (50 μl, 401 μmol) were added to the reaction vial. The reaction vial was filled with nitrogen and heated at 80°C for 30 min. During the silylation reaction, the mixture was sonicated for 5 min. After the reaction was complete, the mixture was evaporated to dryness and anhydrous hexane (100 μl) was added. A sample (1 μl) of the silylated alditol or sugar was directly injected into the gas chromatograph or the gas chromatograph–mass spectrometer. It should be noted that employing this procedure, the silylated alditols or sugars were quite stable. For analysis and sample handling, care was taken to remove traces of water by rinsing syringes and other apparatus with anhydrous hexane.

RESULTS AND DISCUSSION

The gas chromatography of a number of derivatized monosaccharide was accomplished on a DB-1 column to determine if a general monosaccharide chemical structure-retention index relationship (*I*) was present. That an apparent chemical structure–retention index relationship was observed suggested that the structure of unknown derivatized monosaccharides could be predicted from the relationship developed and ultimately assist in the identification of monosaccharides.

To prepare monosaccharides or mixtures of monosaccharides derived from biological or chemical sources of analysis by gas chromatography we developed three micro-scale monosaccharide derivatization procedures: acetylation, permethylation and silylation. While a number of methods have been previously published outlining these derivatizations of monosaccharides [20–24], our methods were especially useful for analysis of extremely low amounts of monosaccharides from biological samples. Each of the derivatization procedures was carried out in a single reaction vessel and loss of the derivatized monosaccharides was thereby minimized. Because the derivatized monosaccharides were somewhat volatile, techniques were developed to perform derivatization chemistry, evaporate the products to dryness and reconstitute in a minimal amount of solvent for analysis with a minimal loss of product. In this manner, analysis sensitivity of monosaccharides was increased to the nmol or pmol level. Thus, acetylation of monosaccharides was performed in the presence of acid- rather than base-catalyzed conditions so that removal of borate (and other salts from the reduction) and acetic acid (from the acetic anhydride) could be accomplished in the wash phase of the reaction and extractive work-up could be avoided.

To permit permethylation, care was taken when working with extremely low levels of monosaccharides because the permethylated derivatives were volatile. Using the procedure outlined under Experimental, and avoiding unnecessary sample transfer, permethylted monosaccharides (*e.g.*, glucose and others) were routinely detected by gas chromatography–mass spectrometry at the level of 50 pg primarily because of the quality of the chromatogram.

As a final method to examine monosaccharide

TABLE I

I VALUES FOR ALDITOL ACETATES

Compound	Formula[a]	*I*
L-Fucitol pentaacetate	$C_{16}H_{24}O_{10}$	1825
[^{2}H]Lyxitol pentaacetate	$C_{15}H_{21}DO_{10}$	1826
Arabitol pentaacetate	$C_{15}H_{22}O_{10}$	1823
[^{2}H]Frucitol hexaacetate	$C_{18}H_{25}DO_{12}$	2056 (2063)[b]
[^{2}H]Mannitol hexaacetate	$C_{18}H_{25}DO_{12}$	2053
[^{2}H]Glucitolamine hexaacetate	$C_{18}H_{26}DNO_{11}$	2210
[^{2}H]Ribitol pentaacetate	$C_{15}H_{21}DO_{10}$	1819
[^{2}H]Galacitol hexaacetate	$C_{18}H_{25}DO_{12}$	2069
[^{2}H]Allitol hexaacetate	$C_{18}H_{25}DO_{12}$	2025
[^{2}H]Glucitol hexaacetate	$C_{18}H_{25}DO_{12}$	2060
L-Sorbitol hexaacetate	$C_{18}H_{26}O_{12}$	2061 (2071)[b]

[a] D = Deuterium.
[b] Two peaks were observed.

chemical structure–retention index relationships we studied silylation of monosaccharides and alditols. To permit silylation without the removal of borate and other salts we used BSA together with TMCS. Hexane was added to the reaction mixture to precipitate the salts and to extract the silylated product into the organic phase. The silylated products were much more stable in anhydrous hexane (*i.e.*, with traces of moisture removed). The excess BSA and TMCS reagents do not interfere with the analysis because these reagents decompose at high temperature.

TABLE II

I VALUES FOR PERMETHYLATED ALDITOLS

Compound	Formula[a]	*I*
L-Fucitol permethylated	$C_{11}H_{24}O_5$	1360
[^{2}H]Lyxitol permethylated	$C_{10}H_{21}DO_5$	1304
Arabitol permethylated	$C_{10}H_{22}O_5$	1304
[^{2}H]Frucitol permethylated	$C_{12}H_{25}DO_6$	1484
[^{2}H]Mannitol permethylated	$C_{12}H_{25}DO_6$	1487
[^{2}H]Ribitol permethylated	$C_{10}H_{21}DO_5$	1267
[^{2}H]Galacitol permethylated	$C_{12}H_{25}DO_6$	1507
[^{2}H]Allitol permethylated	$C_{12}H_{25}DO_6$	1422
[^{2}H]Glucitol permethylated	$C_{12}H_{25}DO_6$	1484
L-Sorbitol permethylated	$C_{12}H_{26}O_6$	1491

[a] D = Deuterium.

TABLE III

I VALUES FOR SILYLATED ALDITOLS

Compound	Formula[a]	*I*
L-Fucitol TMS	$C_{21}H_{54}O_5Si_5$	1832
[^{2}H]Lyxitol TMS	$C_{20}H_{51}DO_5Si_5$	1749 (1753)[b]
Arabitol TMS	$C_{20}H_{52}O_5Si_5$	1748 (1756)[b]
[^{2}H]Frucitol TMS	$C_{24}H_{61}DO_6Si_6$	1989 (1995)[b]
[^{2}H]Mannitol TMS	$C_{24}H_{61}DO_6Si_6$	1988
[^{2}H]Glucitolamine TMS	$C_{24}H_{62}DNO_5Si_6$	2038 (1962)[b]
[^{2}H]Ribitol TMS	$C_{20}H_{51}DO_5Si_5$	1758
[^{2}H]Galacitol TMS	$C_{24}H_{61}DO_6Si_6$	N.R.[c]
[^{2}H]Allitol TMS	$C_{24}H_{61}DO_6Si_6$	1989
[^{2}H]Glucitol TMS	$C_{24}H_{61}DO_6Si_6$	2032
L-Sorbitol TMS	$C_{24}H_{62}O_6Si_6$	1997

[a] D = Deuterium.
[b] Two peaks were observed.
[c] N.R. = Not reported, multiple peaks were observed.

As shown in Table I, the alditol pentaacetates have *I* values around 1820 and the hexaacetates have *I* values around 2050. The alditolamine hexaacetate has an *I* value around 2210. As shown in Table II, the permethylated alditols have *I* values from 1267 to 1507. In the presence of borate, it was very difficult to permethylate the alditols at low level. Glucitolamine (100 μg) was resistant to permethylation. As shown in Tables III and IV, we determined

TABLE IV

I VALUES FOR SILYLATED MONOSACCHARIDES

Compound	Formula[a]	*I*
L-Fucose TMS	$C_{18}H_{44}O_5Si_4$	1751 (1708)[b]
[^{2}H]Lyxose TMS	$C_{17}H_{41}DO_5Si_4$	1685 (1641)[b]
Arabose TMS	$C_{17}H_{42}O_5Si_4$	1748 (1656)[b]
[^{2}H]Fructose TMS	$C_{21}H_{51}DO_6Si_5$	1865 (1852, 1845)[c]
[^{2}H]Mannose TMS	$C_{21}H_{51}DO_6Si_5$	1950 (1902)[b]
[^{2}H]Glucosamine TMS	$C_{21}H_{52}DNO_5Si_5$	1990 (1957)[b]
[^{2}H]Ribose TMS	$C_{17}H_{41}DO_5Si_5$	N.R.[d]
[^{2}H]Galactose TMS	$C_{21}H_{51}DO_6Si_5$	N.R.[d]
L-Glucose TMS	$C_{21}H_{52}O_6Si_5$	1930 (2009)[b]
L-Sorbose TMS	$C_{21}H_{52}O_6Si_5$	1828 (1899)[b]
Maltose TMS	$C_{36}H_{86}O_{11}Si_8$	2516 (2805)[b]
Lactose TMS	$C_{36}H_{86}O_{11}Si_8$	2852 (2720)[b]

[a] D = Deuterium.
[b] Two peaks were observed.
[c] Three peaks were observed.
[d] N.R. = Not reported, multiple peaks were observed.

TABLE V

MASS SPECTROMETRIC PROPERTIES OF DERIVATIZED MONOSACCHARIDES

Compound	Formula[a]	Mol. wt.	m/z[b]
[^{2}H]Glucitol hexaacetate	$C_{18}H_{25}DO_{12}$	435	453 (100)[c], 376 (25), 351 (4), 335 (4), 313 (5), 77 (32)
[^{2}H]Glucitolamine hexaacetate	$C_{18}H_{26}DNO_{11}$	434	435 (100), 420 (7), 375 (84), 324 (20), 141 (56), 112 (40), 97 (55), 83 (62)
Glucose permethylated	$C_{11}H_{32}O_6$	250	268 (22)[c], 236 (100), 219 (10), 187 (67), 172 (25), 157 (10), 140 (64), 88 (21)
[^{2}H]Glucitol TMS	$C_{24}H_{61}DO_6Si_6$	615	616 (66), 544 (2), 436 (2), 346 (3), 320 (11), 217 (10), 164 (18), 132 (22), 117 (11), 90 (100)
Glucose TMS[d]	$C_{21}H_{52}O_6Si_5$	540	558 (19)[c], 541 (3), 468 (5), 378 (2), 361 (73), 288 (20), 198 (52), 90 (100), 588 (76)[c], 541 (5), 468 (5), 451 (5), 378 (2), 361 (76), 288 (20), 198 (57), 90 (100)
	$C_{21}H_{52}O_6Si_5$	540	268 (12)[c], 236 (100), 219 (10), 187 (62), 172 (21), 157 (8), 140 (60), 88 (16)

[a] D = Deuterium.
[b] Determined using chemical ionization mass spectrometry. Relative intensities in parentheses.
[c] A strong ion $[M + NH_4]^+$ was observed.
[d] Two epimers were observed and mass spectral data for each are listed.

the I values for silylated alditols and monosaccharides, respectively. The silylated alditols had I values from 1748 to 2038. The silylated monosaccharides had I values from 1751 to 2852.

To confirm the structure of the derivatized monosaccharides the products were routinely subjected to gas chromatography–mass spectrometry. As shown in Table V, the derivatized monosaccharides possessed characteristic molecular ions and fragmentation patterns for the expected products.

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