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Role of Recent NMR Techniques for Structure Determination of Milk Oligosaccharides: A Review

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Abstract

Carbohydrates are one of the most dominant solid fractions of milk, and besides the main milk sugar (lactose) a number of more complex free oligosaccharides are also present in milk. A broad range of oligosaccharides and their derivatives act as an effective drug against most of acute and chronic diseases, and play an essential role in many molecular processes impacting eukaryotic biology and diseases and exhibit varied biological activity such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anticancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities. In recent years due to modern chromatographic techniques (various forms of HPLC like ion-exchange, size-exclusion, high performance, reverse phase etc.) and spectroscopic techniques like ^{1}H NMR, ^{13}C NMR, 2D-NMR and Mass spectrometry (FAB, MALDI and ES-MS), the isolation as well as structure elucidation of complex oligosaccharides and determination of their anomericity, ring sizes and absolute configuration is very easy task. This current review attempts to summarize role of recent techniques like 1D-NMR (^{1}H, ^{13}C) 2D-NMR (COSY, TOCSY, HSQC and HMBC) for structure interpretation of milk oligosaccharides.

Keywords: Oligosaccharides, NMR Spectroscopy.

Introduction

Today, NMR has become a sophisticated and powerful analytical technology that has found a variety of applications in many disciplines of scientific research, medicine, and various industries. Modern NMR spectroscopy[1,2] has been emphasizing the application in biomolecular systems and plays an important role in structural biology. NMR spectroscopy is one of the leading technologies for the structure determination of oligosaccharide. The NMR spectra of oligosaccharides gave important information about the protons and carbons present in the oligosaccharides. 2D NMR spectroscopy provides actual, high quality and well interpretable data of the sugar molecule. Chemical shift correlation maps obtained by 2D NMR experiments are found to be extremely useful in the identification of components of oligosaccharides, without relying on the analogy with any reference data. There are two fundamental types of 2D NMR spectroscopy. The first is correlated spectroscopy, in which both frequency axis contain chemical shift information and the other is J resolved spectroscopy in which one frequency axis contains spin coupling (J) and other chemical shift (δ) information. Salient features of two-dimensional NMR spectroscopy applied to oligosaccharides are described in this paper.

Aim of the Study

Oligosaccharides exhibit several biological activities such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anti-cancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities. Therefore, to interpret structure of oligosaccharides, this review attempts to summarize role of recent techniques like 1D-NMR (^{1}H, ^{13}C) 2D-NMR (COSY, TOCSY, HSQC and HMBC) and Mass spectrometry (FAB, MALDI and ES-MS) for structure elucidation of milk oligosaccharides.

Methods for Isolation of Milk Oligosaccharides

Generally, oligosaccharides from the milk of different origins are extensively fractionated and isolated by most conventional processing techniques which are

1. KOBATA and GINSBURG[3],
2. URASHIMA et.al.[4],

3. SMITH et.al.[5],
4. EGGE et.al.[6],
5. WEIRUSZESKI et.al.[7],
6. Modified Method of KOBATA and GINSBURG[8]

ChromatographicTechniques Required For Purification of Milk Oligosaccharides

Various purification methods were used during the isolation and purification of milk oligosaccharides and they have been discussed as under-

1. Thin Layer Chromatography9-13
2. Paper Chromatography14,15
3. Column Chromatography16
4. High PerformanceLiquid Chromatography[17-26]

Nuclear Magnetic Resonace (NMR) Spectroscopy in the Analysis of Milk Oligosaccharide[27-40]

Oligosaccharides are a group of monosaccharides with vast structural and chemical diversity; andlimited chemical shift dispersion in NMR spectra makes their study by NMR challenging and intriguing. The assignments of ^{1}H and ^{13}C resonances to their corresponding protons and carbon atoms are usually achieved using a combination of 1D and 2D-NMR experiments such as homonuclear^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSYexperiments and heteronuclear^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC. To elucidate the structure of oligosaccharides following information must be required.

Number of Sugar Residues[41]

In structural analysis of an oligosaccharide, the assignment of anomeric proton chemical shift is of utmost important. The anomeric proton resonances are found in the shift range of δ4.2-5.5 ppm in ^{1}H NMR. Additionally, the number of anomeric C-1 resonances present in a ^{13}C NMR spectrum confirms the number of monosaccharide unit in oligosaccharide molecule. The range of anomeric carbon varies from δ90 – 110 ppm.

Constituents Monosaccharides

The identification of monosaccharide residue is very important in the structure determination of oligosaccharides. The chemical shift data of ^{1}H and ^{13}C NMR together becomes very useful in the identification of monosaccharide unit present in oligosaccharide.

Anomeric Configuration[42,43]

In carbohydrate chemistry, it has been established that “Half of sugar chemistry rotates around the anomericcentre”. In oligosaccharide molecule normally aα-anomer resonates downfield compared to the β-anomer in Dpyranoses in $^{4}C_{1}$ conformation. If H-1 and the H-2 are both are in an axial configuration in pyranose structure, a large coupling constant (8-10 Hz) is observed, whereas if they have equatorial-axial configuration, this is smaller ($J_{1,2}$ ~4 Hz), and for equatorial-equatorial oriented protons, even smaller coupling constants are observed (<2 Hz). The ^{13}C chemical shift for anomeric configuration of sugar residue appears in δ90-110 ppm, but most importantly the one bond ^{13}C-^{1}H coupling constants in pyranose can be used to determine the anomeric configuration unequivocally.

Linkages and Sequence[43]

The ^{1}H and the ^{13}C chemical shift may give an indication for the linkage of complete oligosaccharide moiety. The effect of glycosylation shift depends on the linkage type and the changes in the chemical shift are in general larger at the glycosylation site than at neighboring positions. A HMBC and Inter residue NOEs experiment may give information about the glycosidic linkages. Position of linkage could be well defined by a comparable study of ^{1}H assignment of natural and acetylated oligosaccharide. Moreover 2D experiments of Homo and Hetero nuclear experiments like COSY, TOCSY and HMBC gives relevant information.

Position of Appended groups[44,45]

The attachment of a non-carbohydrate group like a methyl, acetyl, sulfate or a phosphate group could be pointed by the proton and carbon NMR chemical shifts. Attachment of these groups affects the proton and carbon resonance where the group is located. Normally downfield shifts of ~δ0.2-0.5 ppm are observed for protons and δ5-10 values for ^{13}C. This places these resonances in a less crowded area of the spectra and helps the identification of novel residues.

Structure Reporter Group[46-48]

Sincethe NMR data of oligosaccharide are highly complex,Vligenthart et.al. introduced the “structural reporter group” (SRG) concept, which was based on signals outside the bulk region (δ 3-4) in the ^{1}H NMR spectra of the oligosaccharide. This structural reporter group concept helped in the identification of novel residues and characterization of oligosaccharides. Moreover they are NMR fingerprints of a particular linkage or group.

^{1}H and ^{13}C NMR experiments

^{1}H-NMR spectroscopy is often the first step in structural studies of oligosaccharides by NMR. The number of sugar residues can be estimated by integration of the signals in the region of the anomeric protons. The number of anomeric resonances in the ^{13}C-NMR spectrum will further confirm these results. ^{1}H and ^{13}C experiments can also give some indication about the linkage and sequence of the sugar residues through changes in chemical shifts, but in general both homo- and heteronuclear 2D-NMR experiments are required for complete assignmentof oligosaccharides. If the anomeric signals are well resolved they appear as doublets from which the $^{3}J_{H1,H2}$coupling constants can be obtained, and give information about the anomeric configuration. ^{13}C-NMR is much less sensitive than ^{1}H NMR due to the low natural abundance of the ^{13}C nucleus and the fact that the gyromagnetic ratio is only ¼ of that of ^{1}H, but the ^{13}C spectra show a greater dispersion of chemical shifts.

In the ^{1}HNMR Spectroscopy[49-56], a high resolution ^{1}H-NMR spectra gives valuable information about milk oligosaccharide's structure. The chemical shift of a particular anomeric proton and its splitting pattern gives an idea of the monosaccharide units present; simultaneously it also fixes the configuration of sugar linkage and conformation of that monosaccharide unit. The proton NMR spectroscopy

of oligosaccharides suffers from severe spectral overlap, because most of the monomeric residues differ only in their stereochemistry and their magnetic properties are only little influenced by their position in chain. The chemical shift of anomeric protons and methine protons of different sugars are confined to the region δ 4.3-5.5 and δ 3.0-4.2 respectively hence it requires expert interpretation of spectra for monosaccharide identification. The analysis of reducing oligosaccharides showed that the anomeric configuration of the reducing end sugar also exerts its influence on the spectral parameters of residues in its spatial neighborhood, being sometimes even the non-reducing end sugar. In D-pyranoses $^4C_1$ conformation the α-anomer resonates downfield in comparison to β-anomer. The chemical shift value for α-anomer lied in the range 4.9-5.4 ppm and for β-anomer it lied in the region 4.4-4.8 ppm. The α-anomeric doublet showed coupling constant J=3-4 Hz whereas the β-anomeric doublet showed J value of 6-9 Hz. All these values were correlated with known structures to yield relevant information in terms of monosaccharides units and their relative abundance. The structure of different linkages can be defined in terms of NMR parameters of their structural reporter groups. In case of milk oligosaccharides the anomeric proton resonances are found in the chemical shift range 4.3-5.5 ppm and the remaining ring proton resonance are found in the range 3.0-4.2 ppm. But in case of acetylated oligosaccharides acetyl groups induce a strong downfield shift of proton which directly linked to acetylated carbons. Hence the signals of methine protons and methylene protons occur downfield in the region of 4.0-4.8 ppm. The resonances of protons linked to the non-acetylated carbons at the site of glycosidic linkage and at the ring C-5 occur in the chemical shift range between 3.5 and 3.9 ppm. To resolve the spectral complexities of oligosaccharides, Vligenthart et.al.introduced the "structural reporter group" concept, which was based on signals outside the bulk region (δ 3-4) in the ^{1}H-NMR spectra of the oligosaccharide. This approach is used to identify individual sugars or sequence of residues. These structural reporter groups include anomeric proton, equatorial protons, deoxy protons and that distinct functional group such as amide group. ^{1}H-NMR gives anomeric protons at 4.3-5.9 ppm, methyl doublets of 6-deoxy sugars at 1.1-1.3 ppm, methyl singlet of acetamido groups at 2.0-2.2 ppm and various others with distinctive chemical shift. Some of the common spectral feature of the ^{1}H-NMR structural reporter groups of milk oligosaccharides are summarized below

1. In the ^{1}H-NMR spectra the reducing Glc residue is characterized by the H-1 signals for its α and β anomers at δ5.221 ($J_{1,2}$ 3.7 Hz) and δ 4.688 ($J_{1,2}$ 8.0 Hz) respectively with ratio of 7:10.
2. The 4-substituted reducing Glc shows anomeric signals for both the α- and the β- anomeric at δ 5.22 and 4.66 ppm, with H-2 of the β-from in the range of δ3.2-3.3 ppm as triplet.
3. The 3,4-disubstituted reducing Glc shows anomeric signals from both the α- and the β-anomeric at δ 5.22 and 4.66 ppm, with H-2 of the β-from at a typical downfield shift above δ3.35 ppm.
4. The 3-substituted β-linked Gal shows signal for H-1 at 4.4 ppm and H-4 of β-linked Gal showed at a typical downfield shift around δ 4.13-4.15 ppm due to substitution at the 3-position.
5. The H-4 of (1→6) linked β-Gal appeared at δ 3.8-3.9 ppm and H-4 of (1→3) linked β-Gal at δ 3.9-4.2 ppm.
6. Signal for H-1 of the unsubstituted Gal residue appears around 4.44-4.47 ppm.
7. β-linkedGlcNAc residues with anomeric signals appear at δ 4.6-4.7 ppm and CH_3 signals in the range of δ 2.02-2.08 ppm. H-1 of the (1→6) linked GlcNAc appears at lower chemical shift value (δ 4.6 ppm.) than the (1→3) linked GlcNAc residue (4.7ppm). A splitting of the anomeric doublets is due to the anomerization of the reducing terminal.
8. The H-2 of β-GlcNAc appeared at 3.6-3.8 ppm and H-2 of β-GalNAc appeared at 3.8-4.2ppm.
9. Presence of anomeic signal with a integration of two proton at 4.44-4.6 ppm suggest a LNT structure in which one β-Gal is attached to Glc by (1→4) linkage while another β-Gal unit is attached to β-GlcNAc or β-Glc by (1→3) linkage i.e. β-Gal(1→3) β-GlcNAc(1→3/6) β-Gal (1→4) Glc or β-Gal(1→3) β-Glc (1→3/6) β-Gal(1→4) Glc moieties is present.
10. α-linked Gal residue appeared at δ4.94-5.2 ppm. The (1→4) linked α-Gal residues showed anomeric signal at δ 5.02 ppm, (1→2) linked α-Gal residues showed anomeric signal at δ 5.20 ppm and (1→3) linked α-Gal residues showed anomeric signal between δ 5.02-5.20 ppm.
11. α-linked Fuc residues anomeric signals appeared at δ5.02-5.43 ppm. The presence of fucose subunit could be inferred by the presence of CH_3 doublet at δ 1.1-1.3, H-5 at δ 4.2-4.9 and the anomeric doublet at δ5.02-5.4 ppm.
12. Generally (1→4) linked fucose occur near δ4.98ppm, (1→2) linked fucose occur near δ5.38ppm and (1→3) linked fucose occur between the two.
13. The presence of sialic acid residue could be ascertained by the characteristic resonances of H-3 axial and equatorial protons at 1.78 and 2.75 ppm respectively. The location of Neu5Ac residue can be deduced as follows. (a) the signal for H-3a and H-3e of Neu5Ac residue can be used to discriminate between (2-3) and (2-6)-α-linkage to Gal. (b) for an α-Neu5Ac(2-3)-β-Gal-(1- sequence, the signal for H-3 of Gal residue is shifted downfield by 0.6 ppm of the ring protons.

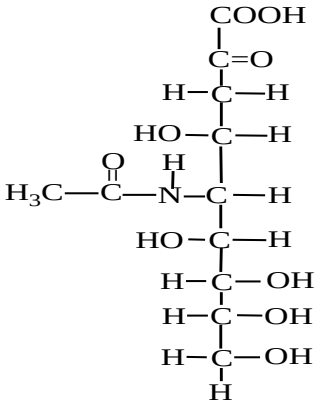
^{13}C-NMR spectroscopy[57-65] has been extensively used to assign the conformation and type of anomeric linkages in Oligosaccharides. The ^{13}C-NMR spectroscopy also has enormous potential in oligosaccharides structure determination because its greater chemical shift dispersion and lack of complexities arise from spin-spin coupling and overlapping resonances, also with those arising from solvents. In contrast to the rather crowded and poorly

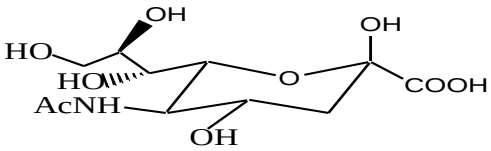
resolved ^{1}H-NMR spectrum, the proton noise decoupled ^{13}C-NMR is usually well resolved and has few overlapping lines and therefore is inherently easy to interpret but difficult to assign chemical shifts to specific carbon due to small differences in chemical shift among ring carbons of each monomeric residue as well as from different monomeric residue.The appearance of anomeric resonances in a well separated chemical shift range of 90-110 ppm help greatly in determining the number of O-linked monosaccharides. The C-1 of reducing end residue appears in the region 90-98 ppm and other non-reducing monosaccharide units appear at 98-110 ppm[66]. The rest of methine and methylene resonances absorb between 51-86 ppm. The appearance of methine resonances between 52-57 ppm[66] is generally associated with amino substituted carbon signals at an amino sugar residue. The low field absorption in the region 170-176 ppm reflects the presence of a carboxylic group of hexapyranoic acids or the carbonyl group of acetamido sugars. The presence of an acetaamido sugar may further be complemented by the appearance of methyl resonances in the region 20-24 ppm. The spectral region between 57-64 ppm contains signals for all the unsubstitutedhydroxy methylene resonances C-6, whereas methyl resonances of 6-deoxy sugars generally appear in the region 16-19 ppm.

Since naturally occurring monosaccharides are generally hexoses or pentoses therefore, each hexose and pentose unit introduces either six or five resonances, respectively. Accordingly in a well-resolved ^{13}C-NMR spectrum, in most cases the number of monosaccharide residues can be easily ascertained by simply dividing the total number of signal absorbing between 60-85 ppm either by five or four or by combination of both. In a hexose monosaccharide besides the anomeric signal it give rise to five resonances whereas in case of 6-deoxy hexose and pentose it give rise to four resonances in the above mentioned chemical shift range. The coupling pattern for GalNAc and GlcNAc in ^{1}H-NMR is similar to Gal and Glc respectively but in ^{13}C NMR an upfield shift of $\delta_{C2\alpha}$~55.4, $\delta_{C2\beta}$~58ppm for GlcNAc and an upfield shift of $\delta_{C2\alpha}$~51.4, $\delta_{C2\beta}$~54.9ppm for GalNAc has been reported. In the chemical shift analogy method the chemical shifts of carbon atoms in identical residues of similar oligosaccharide structure will be influenced only by glycosylation shifts, primarily by the δ shift (approximately 8 ppm downfield) for a substituted carbon atom and secondarily by the β shift (1-2 ppm upfield) for those carbon atoms adjacent to the linkage position. The ^{13}C chemical shift reveals the anomeric configuration in a manner similar to the proton chemical shift but most importantly the one bond ^{13}C-^{1}H coupling constant in pyranoses can be used to determine the anomeric configuration. For D sugars in the $^{4}C_{1}$ conformation a $J_{C1,H1}$~170 indicates an α-anomeric sugar whereas $J_{C1,H1}$~160 indicates an β-anomeric sugar configuration.

The presence of sialic acid residue could also be well determined by ^{13}C NMR spectroscopy. The anomeric signals (C-2) appear at δ 100-101 ppm while signal for –COOH group appears at δ 174 ppm[67].

N-acetyl Neuraminic acid





(5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid)

Two Dimensional NMR Spectroscopy

2D-NMR spectroscopy provides actual, high quality and well interpretable data of the sugar molecule. There are two fundamental types of 2D-NMR spectroscopy. The first is correlated spectroscopy, in which both frequency axis contain chemical shift information and the other is J resolved spectroscopy in which one frequency axis contains spin coupling (J) and other chemical shift (δ) information. The salient features of two-dimensional NMR spectroscopy applied to oligosaccharides are described as below-

Correlated Spectroscopy (COSY)[68-71]

Homonuclear through bond correlations, ^{1}H-^{1}H COSY is used to establish the direct neighboring connectivity of protons. The anomeric protons are often used as starting point in the assignment of the ring protons due to the fact that they have well separated chemical shifts and are generally only coupled to one proton. The connectivity between protons within a sugar residue can form this be mapped out via a series of cross-peaks. There can be difficulties in assigning all protons, due to overlapping signals or lack of cross-peaks due to small 3J coupling constants. The clear representation of 2D-

NMR spectrum is obtained as contour plots of mutual coupling which exists between two nuclei (^{1}H-^{1}H, ^{13}C-^{13}C), cross peak appears at the chemical shift coordinates (X, Y) and (Y, X). Therefore, COSY spectra contain information on spin coupling networks within the constituent residues of the oligosaccharide through the observation of cross peaks[72-81]. Assignment of this spectrum by coupling-correlation requires an initial point for the identification of the individual spin systems of sugar rings. The most downfield ^{1}H signals (anomeric) are always a convenient starting point for the assignment. With in typical aldohexopyranosyl ring, the coupling network is unidirectional i.e. H-1 couples to H-2; H-2 couples to H-1 and H-3, H-3 to H-2 and H-4 and so on. However, the presence of no or small coupling between H-4 and H-5 ($J_{4,5}$=2-3 Hz) of galactosyl residue and coupling between H-1 and H-2 in mannopyranosyl residue prevents detection of cross peaks. Thus Cosy experiments and its RELAY extensions give coupling patterns along with shift information , which allow each monosaccharide residue to be identified and designated as α or β and also provide information about sugar identity and substitution pattern.

Total Correlation Spectroscopy (TOCSY)[82]

Homonuclear through bond correlations, ^{1}H-^{1}H TOCSY is related to COSY in the way that cross-peaks of coupled protons are detected. The additional information from the TOCSY spectrum is that there are cross-peaks between (almost) all spins in the spin system. The magnetization is transferred, during the mixing time, to the vicinal coupling partner, and can be further transferred throughout the entire spin system. The magnetization transfer can be interrupted by small 3J coupling constants. This property is advantageous when deducing the configuration to be manno, galacto or gluco. The number of transfer steps can be adjusted by changing the mixing time, a mixing time of 20 ms (one step transfer) will give essentially the same information as the COSY experiment, whereas a mixing time of 80-120 ms will give a five to six steps transfer. Thus TOCSY can give total correlation of all protons in a chain with each other and serve for the identification of single residue in oligosaccharides. H. Kogelberg et.al. used the TOCSY technique in structural elucidation of octasaccharide isolated from human milk. Thus TOCSY spectrum of acetylated sugar gives complete information regarding the position of glycosidic linkage in a particular monosaccharide unit. However the sequence of cross peaks in TOCSY spectrum could be confirmed by COSY spectrum of same sample**.** It also provides the information for Carbon and protons involved in the glycosidic linkages.The anomeric proton/ carbon cross peaks lies in the range of δ4.2-5.5 ppm and δ90-112 ppm respectively. However, Cross peak of proton and carbon for the position involve in the glycosidic linkage lies between δ70-80 ppm for carbon and δ3.5-4.2ppm for proton.

Heteronuclear single quantum coherence (HSQC)[83]

Heteronuclear through-bond correlations, ^{1}H-^{13}C HSQC provides proton carbon coupling across a single bond and correlates the protons with the directly bonded carbons, through one bond couplings. The cross-peaks contain information about the chemical shifts of the corresponding protons and carbons. The number of monosaccharides can more easily be determined in an HSQC spectrum than in a ^{1}H spectrum due to the added dispersion of chemical shifts also in the carbon dimension. The HSQC spectrum gives information about cross peak of anomeric and ring proton to their directly attached carbon atom with linkages between monosaccharides in oligosaccharide moiety.

Heteronuclear multiple bond correlation (HMBC)[84]

^{1}H-^{13}C HMBC shows cross-peaks between protons and carbons that are two or three bonds away. With this experiment it is possible to study quaternary carbons that were not visible in the HSQC experiment. The HMBC experiment is used to establish the linkage between monosaccharide units via the glycosidic bond. The protons at acetyl-protected position now show a three bond ^{13}C-^{1}H coupling and can be easily detected by HMBC experiment and thus position of glycosidic linkage was confirmed. The sensitivity of heteronuclear multiple bond correlation (HMBC) is increased by the use of ^{13}C labeled acetic anhydride, and the assignments can be readily identified. Thus with the help of HMBC experiment, the correlation of proton with adjacent carbon can be achieved and this information is very useful in structural elucidation of oligosaccharides.

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