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Review

Three-dimensional mapping of N-linked oligosaccharides using anion-exchange, hydrophobic and hydrophilic interaction modes of high-performance liquid chromatography

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Abstract

To determine the structure of N-linked oligosaccharides, a three-dimensional (3-D) sugar mapping technique for pyridylaminated neutral and sialyl oligosaccharides is proposed. The pyridylaminated oligosaccharide mixture is first separated by HPLC on a diethylaminoethyl anion-exchange column and the elution data are placed on the *Z*-axis. Neutral and mono-, di-, tri- and tetrasialylloligosaccharides are then individually separated on both a hydrophobic octadecylsilylsilica column and a hydrophilic amide-silica column under the same conditions as described previously for neutral oligosaccharides. The validity of the 3-D mapping technique was tested using sialyl pyridylaminated oligosaccharides from human serum glycoproteins.

Contents

1. Introduction

Recent developments in elaborate chromatographic techniques allow a new approach to the determination of carbohydrate structure with or without derivatization [1,2]. One of the most promising and popular precolumn derivatization methods is to label the reducing ends of the oligosaccharides with 2-aminopyridine to provide fluorogenic properties [3,4]. Sometimes liquid chromatography is carried out in two different column–solvent combinations, resulting in "two-dimensional (2-D) mapping" [5,6]. The most extensive mapping method involves the accumulation of HPLC data for 2-pyridylaminated oligosaccharides (PA-oligosaccharides) chromatographed on an octadecylsilica (ODS-silica) column and on an amide-silica column [7]. N-

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Linked neutral PA-oligosaccharides are separated by HPLC using the two different columns and their elution positions are expressed in terms of glucose equivalent.

To date more than 220 neutral oligosaccharides have been documented on the 2-D map [8,9]. Many N-linked oligosaccharide structures have been determined by use of this method, e.g., the taste-modifying protein miraculin [10], human IgGs [11], rice α-amylase [12], murine lymphocytes [13], the nicotinic acetylcholine receptor from *Torpedo californica* [14], recombinant erythropoietin [15], human urinary kallidinogenase [16], the sphingolipid activator proteins saposins A, C and D [17] and duck ovomucoid [18].

We have now extended this technique to 3-D mapping [19]. This 3-D mapping method permits the direct analysis of not only neutral oligosaccharides but also of the sialyloligosaccharides [20].

2. Outline of procedure, including three-dimensional mapping technique

As shown in Fig. 1, the asparagine-linked oligosaccharides are enzymatically released after the preparation of the glycopeptides. We always use glycoamidase A from almonds. All N-linked oligosaccharides tested so far have been cleaved from glycopeptides by this enzyme, whether they

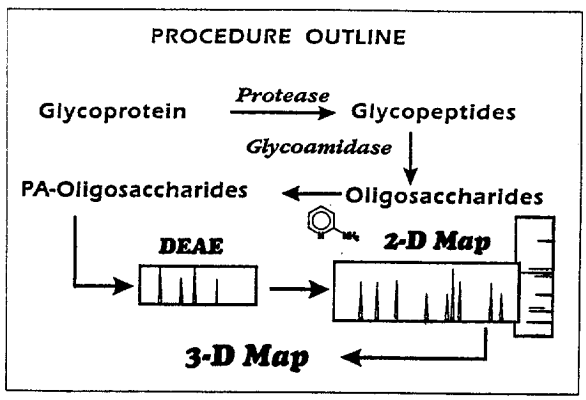

Fig. 1. Scheme for the structural analysis of N-linked neutral and sialyloligosaccharides.

were neutral, sialylated, sulfated or phosphorylated. Naturally, all oligosaccharides recorded on the database of the 2-D or 3-D map [7–9,19,20] have been obtained by glycoamidase A digestion. The reducing ends of the oligosaccharide mixture were then derivatized with a fluorescent reagent, 2-aminopyridine. This improved method for PA derivatization works well for sialylated oligosaccharides and for neutral oligosaccharides [4]; also, because of its fluorescent nature, the sensitivity of detection of PA-oligosaccharides is in the picomole range. The PA-oligosaccharide mixtures were separated on three different HPLC columns.

3. Two-dimensional mapping technique for neutral oligosaccharides

We previously proposed the 2-D sugar mapping technique for elucidation of the structures of N-linked oligosaccharides using PA-derivatized neutral oligosaccharides [7]. This is not only a powerful separation technique but it can also establish oligosaccharide structures from the elution positions on two HPLC columns (Fig. 2) [8]. Separation on the amide column (Y-axis) depends mostly on the molecular size of each oligosaccharide. Separation on the ODS column depends on the fine structure of each oligosaccharide. Therefore, oligosaccharides of the same molecular size and which elute at the same position on the amide column can be separated on the ODS column. The elution positions from these two columns provide a unique set of coordinate values for each PA-oligosaccharide, and many sets of such coordinates make up a 2-D map.

The elution time expressed in time or volume units can be variable, depending on the individual column, its age or the batches of buffers used. The introduction of the glucose unit is meant to reduce such variations. For this, we calibrated both the ODS and amide columns with isomaltooligosaccharide mixtures (Fig. 3a and b). The numbers 4, 5, 6, etc., indicate the degree of polymerization of glucose. Next, a sample PA-oligosaccharide was applied to the columns. In this case, the glucose unit of the sample PA-oligosaccharide is measured as 14.7 on the X-axis and 6.7

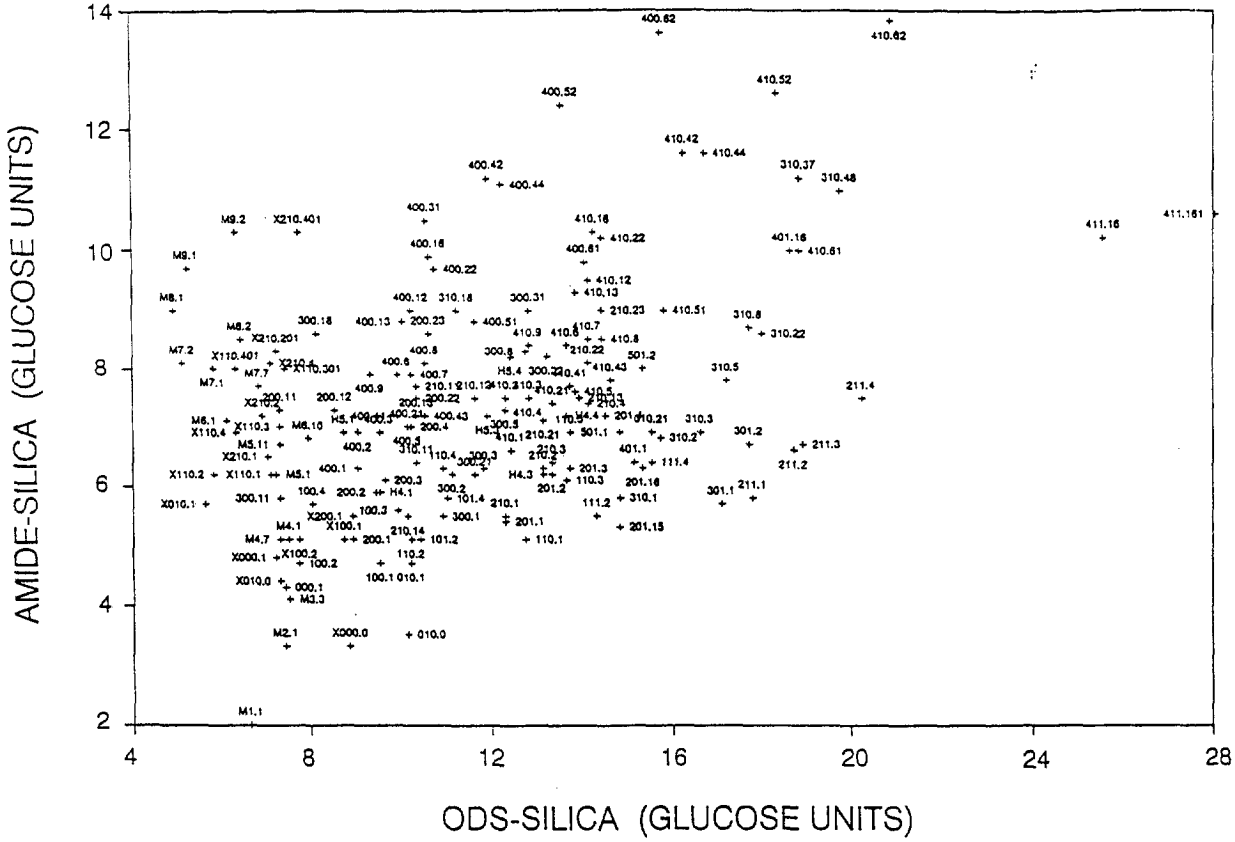

Fig. 2. Two-dimensional sugar map of PA-oligosaccharides expressed as glucose units [8].

on the *Y*-axis. The next step is to plot the coordinates on the 2-D map (Fig. 3c). The glucose unit, 14.7 on the *X*-axis and 6.7 on the *Y*-axis, is plotted on the 2-D map as a coordinate. The structure of an unknown oligosaccharide can be elucidated by comparing its position on the map with the position of the known standards plotted on the 2-D map. Actually, by using a computer search, we choose a couple of candidate PA-oligosaccharides whose coordinates coincide with that of the sample PA-oligosaccharide within an allowable error (±5%).

4. Strategy for the structural determination of an unknown sample on the 2-D map

It is sometimes necessary to go beyond a direct comparison of the elution positions. For example, if the coordinate 9.5 on the *X*-axis and 6.0 on the *Y*-axis of an unknown sample is placed between two known structures 9.6, 6.1 and 9.4, 5.9 (Fig. 4), the strategy for the analysis of the unknown sample is as follows. In many cases, co-injection with a standard PA-oligosaccharide is the most reliable solution. Moreover, a digestion method using several glycosidases is very useful. Although the elution positions of the two candidate oligosaccharides GlcNAcβ2Manα6-(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (9.6, 6.1) and Galβ4GlcNAcβ2Manα6-(GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (9.4, 5.9) are very close, after β-N-acetylhexosaminidase digestion, the elution positions of the resultant two oligosaccharides move away as illustrated in Fig. 4. Then, after sequential digestion of β-galactosidase and β-N-acetylhex-

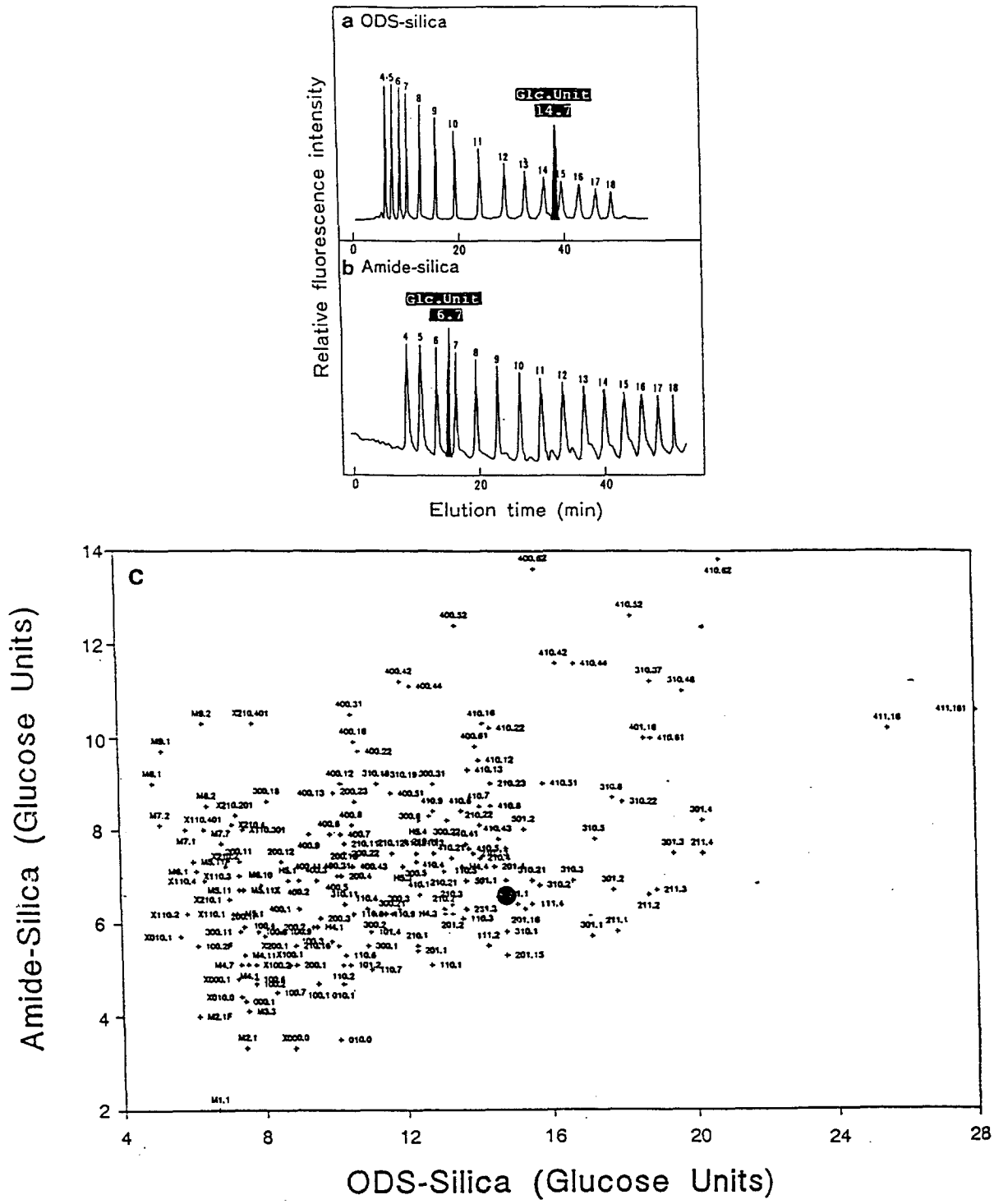

Fig. 3. How to express the elution volume as a glucose unit. Chromatography of PA-derived isomaltooligosaccharide mixture on (a) an ODS-silica and (b) an amide-silica column. Black peaks indicate elution positions of a sample oligosaccharide. (c) The glucose unit 14.7 on the X-axis and 6.7 on the Y-axis is plotted on the 2-D map as a coordinate (black circle).

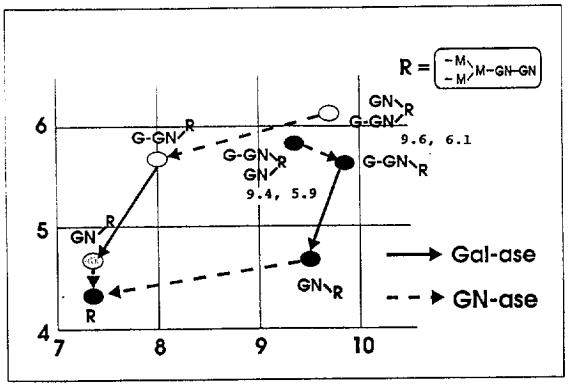

Fig. 4. Strategy for the structural determination of an unknown sample on the 2-D map. Details are described in the text.

osaminidase, the elution position of each standard oligosaccharide finally changes to a common trimannosyl core structure. Therefore, we can establish the originally unknown structure by a series of transformations.

5. Extension to sialyloligosaccharides (3-D mapping technique)

We have more than 220 oligosaccharides on the above-mentioned 2-D map. However, all of these are the neutral oligosaccharides. It is desirable to extend the method to sialyl oligosaccharides.

Fig. 5 shows the steps for constructing a 3-D map for the structural determination of sialyloligosaccharides [19]. First, a PA-oligosaccharide mixture is separated on a diethylaminoethyl (DEAE) column into neutral, mono-, di-, tri- and tetrasialyloligosaccharides according to the sialic acid content of each fraction [20]. Each oligosaccharide separated on the DEAE column is then injected on to an ODS column and the elution volumes are recorded as glucose units, which will form the X-coordinates. Next, each peak from the ODS column is applied to an amide column, and the elution volumes are recorded as glucose units to be plotted on the Y-axis. When all the X- and Y-values are plotted for each sialylation group, a 2-D map is created for each of these groups separated by the DEAE column (Fig. 5a).

It is important to point out that for each 2-D map, the HPLC elution conditions used to obtain the X- and Y-coordinates are identical, so the coordinates can be transposed from layer to layer. If we plot all the coordinates from the separated 2-D maps on a single 2-D map, however, the result is slightly confusing because too many coordinates will be crowded on it (Fig. 5b). Three-dimensional mapping is introduced to alleviate such confusion (Fig. 5c). This consists of layers of 2-D maps. Each of these layers lined up on the Z-axis is essentially a 2-D map for a group of oligosaccharides obtained by separation on a DEAE column based on their sialic acid content. The lowest layer is that for the neutral oligosaccharides, and this in fact is the 2-D map we reported previously [7]. The next layer is that for the monosialyloligosaccharides, followed by those for the di-, tri- and tetrasialyloligosaccharides.

6. Chromatograms of oligosaccharide separation leading to a 3-D map

As an example, we shall briefly describe the structural analysis of N-linked neutral and sialyloligosaccharides derived from human serum glycoproteins [20] (Fig. 6). First, a PA-oligosaccharide mixture is separated on the DEAE column according to the sialic acid content. Neutral and mono-, di- and trisialyloligosaccharides are well separated. The molar ratio of these fractions is about 30, 20, 40 and 10%, respectively. Each of the neutral, mono-, di- and trisialyloligosaccharides is then separated into the many peaks on the ODS column shown in Fig. 6b. The elution volume of each peak is recorded as a glucose unit on the X-axis. Each of the separated oligosaccharides is then applied individually to the amide column, checked for purity and the elution volume of each is recorded as a glucose unit on the Y-axis. In all cases, sample oligosaccharides were found to be homogeneous. The elution volumes from these

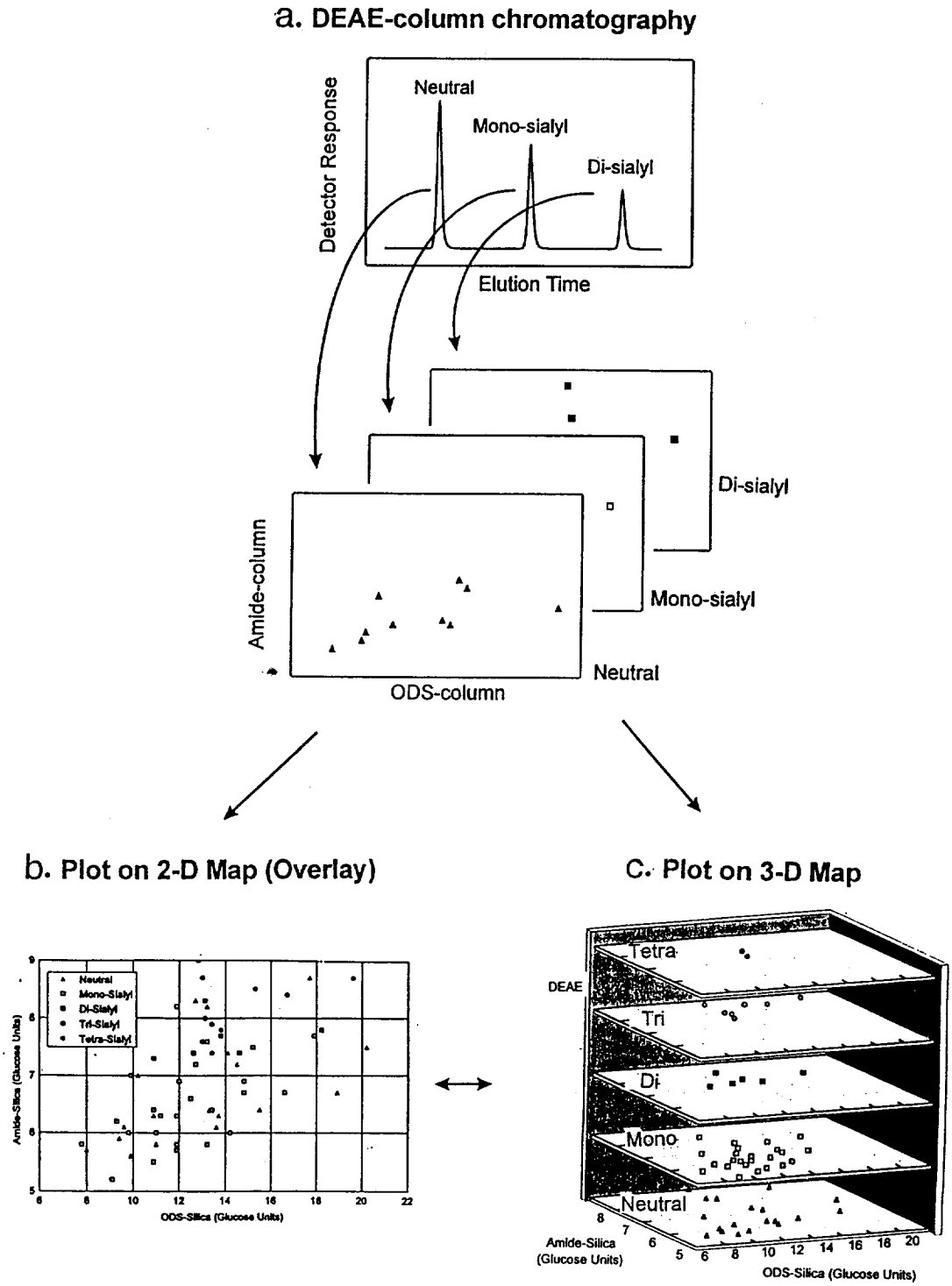

Fig. 5. Transposition of coordinates of PA-oligosaccharides from 2-D to 3-D map [19]. Details are described in the text.

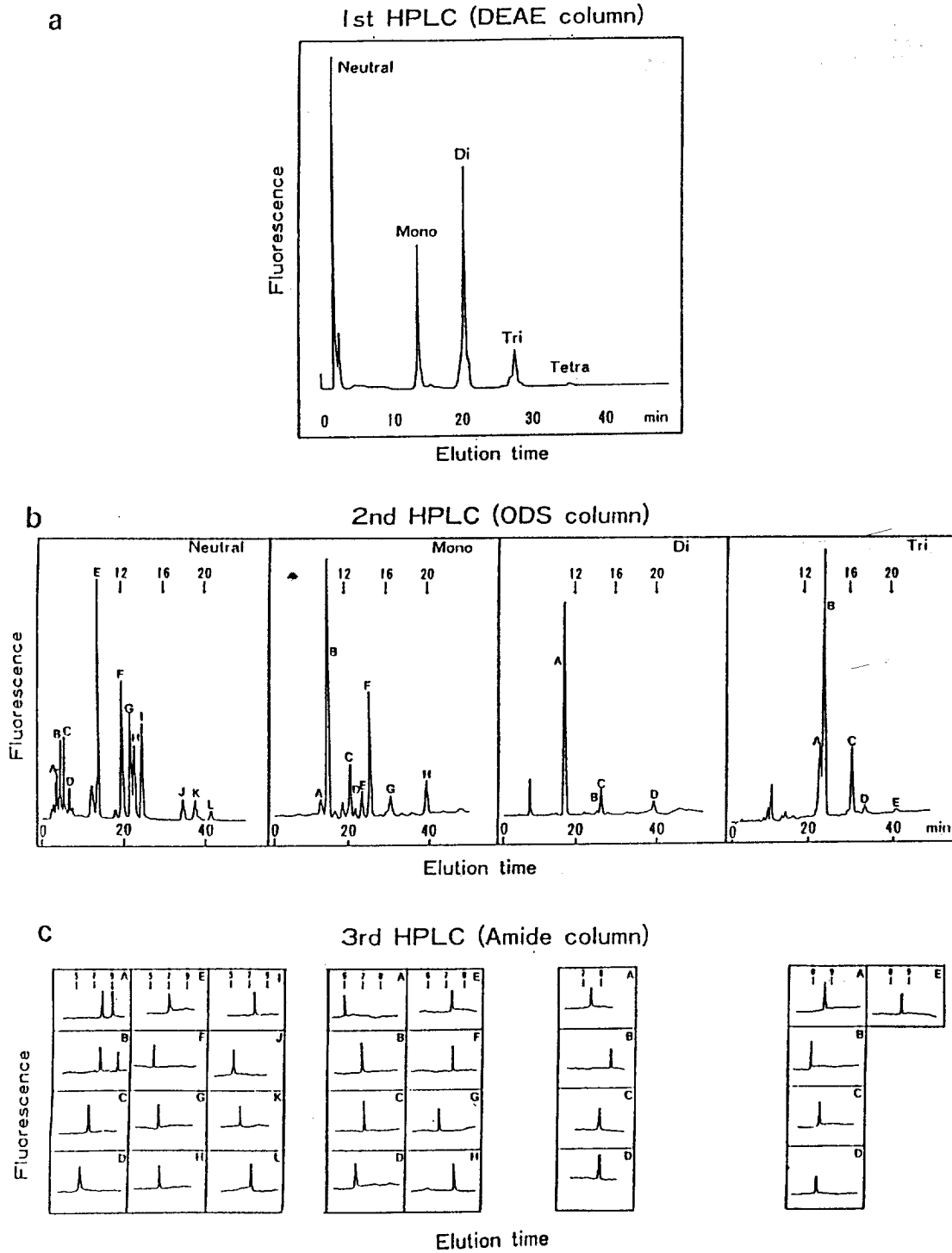

Fig. 6. Elution profile of PA-oligosaccharides from human serum glycoproteins [20]. Details are described in the text.

two columns provide a unique set of coordinates. The coordinates of every oligosaccharide in this sample were coincidental with those of known oligosaccharides, so it was possible to assign a unique structure to each of the unknown oligosaccharides. For example, Table 1 shows the structures of monosialyloligosaccharides from human serum. The main components are bian-

Table 1
Structures and elution positions of PA-derivatized monosialyloligosaccharides obtained from human serum glycoproteins

Code No.	Structure of PA-oligosaccharide	Glc units	
		ODS	Amide
A 1A1-200.3	GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc / Neu5Acα6Galβ4GlcNAcβ2Manα3	9.3	6.2
B 1A1-200.4	Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc / Neu5Acα6Galβ4GlcNAcβ2Manα3	9.9	7.0
C 1A2-200.4	Neu5Acα6Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc / Galβ4GlcNAcβ2Manα3	12.0	6.9
D 1A1-210.3	GlcNAcβ2Manα6 Fucα6 \ | Manβ4GlcNAcβ4GlcNAc / Neu5Acα6Galβ4GlcNAcβ2Manα3	12.5	6.6
E 1A1-201.4	Galβ4GlcNAcβ2Manα6 \ GlcNAcβ4—Manβ4GlcNAcβ4GlcNAc / Neu5Acα6Galβ4GlcNAcβ2Manα3	12.7	7.2
F 1A1-210.4	Galβ4GlcNAcβ2Manα6 Fucα6 \ | Manβ4GlcNAcβ4GlcNAc / Neu5Acα6Galβ4GlcNAcβ2Manα3	13.2	7.6
G 1A3-210.4	Galβ4GlcNAcβ2Manα6 Fucα6 \ | Manβ4GlcNAcβ4GlcNAc / Neu5Acα3Galβ4GlcNAcβ2Manα3	14.8	6.9
H 1A1-211.4	Galβ4GlcNAcβ2Manα6 Fucα6 \ | GlcNAcβ4—Manβ4GlcNAcβ4GlcNAc / Neu5Acα6Galβ4GlcNAcβ2Manα3	18.3	7.7

tennary with a Neu5Acα2→6 residue on the lower branch with or without a fucose residue on the reducing N-acetylglucosamine. We obtained only one oligosaccharide with a Neu5Acα2→3 residue.

7. Conclusion

Using anion-exchange, hydrophobic and hydrophilic interaction modes of HPLC columns, a mixture of neutral and sialyl oligosaccharides is completely separable. Structural assignment of both neutral and sialyl N-linked oligosaccharides is very easy. Even closely related Neu5Acα2→3- and α2→6-containing oligosaccharides can be differentiated on the map. The database on the 3-D map is highly reproducible. Unknown structures whose coordinates do not agree with those of known compounds are easily detected.

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