The effects of ethanol on the glycosylation of human transferrin

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Introduction

Human serum transferrin is a glycoprotein synthesized by the liver and involved in iron transport between sites of absorption and delivery (de Jong et al., 1990). Transferrin contains two N-glycosylation sites at the C-terminal lobe. Their carbohydrate structures consist mainly of sialylated biantennary and triantennary complex N-linked oligosaccharides in the total ratio of 85:15 (Fu and van Halbeek, 1992). The heterogeneity of transferrin glycans has been elucidated by numerous structural studies (Spik et al., 1975, 1985; Marz et al., 1982), and the different combinations of the two glycans result in tetra-, penta-, and hexasialylated variants of serum transferrin.

The presence of a hyposialylated form of transferrin in the plasma of chronic alcohol consumers was described 25 years ago by Stibler and termed carbohydrate-deficient transferrin (CDT). Due to the sensitivity and the specificity of CDT, it is considered the best marker available of chronic alcohol consumption. More than 150 studies have been performed on CDT as a marker of heavy drinking. However, the use of this marker is still controversial (Fagerberg et al., 1994; Schmitt et al., 1998; Allen and Sillanaukee, 1999). The mechanisms of transferrin hyposialylation in chronic alcohol intake are not well understood. Ethanol has been shown to modify both the biosynthesis and the catabolism of glycoproteins (Stibler and Borg, 1991; Ghosh et al., 1993; Xin et al., 1995; Cottalasso et al., 1996; Clemens et al., 1996; Tworek et al., 1996; Lakshman et al., 1999). Therefore, the presence of CDT in the serum of chronic alcohol drinkers should correspond to the sum of all the hyposialylation mechanisms, and the oligosaccharide moiety of transferrin should reflect the involvement of each of them. Conversely, the only modification of carbohydrate deficient transferrin described in the literature is the loss of one or both glycan chains (Landberg et al., 1995; Peter et al., 1998; Inoue et al., 1999).

The goal of the present work was to identify the structure of the oligosaccharides present on each isoform of transferrin. The knowledge of the involvement of the various hyposialylation mechanisms might be of importance in the understanding of the presence or the absence of the hyposialylated transferrin during chronic alcohol intake as well as in determining if this marker can be used to distinguish signs of alcohol consumption and of alcohol related damage.

Results

Purification of transferrin isoforms from healthy subjects

Human transferrin was isolated from the serum in a single-step procedure using immunoaffinity chromatography on immobilized rabbit anti-human transferrin IgG column. The purity of the retained fraction was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectrofocusing (IEF). SDS–PAGE demonstrated the presence of a single broad band of transferrin with no contaminating serum glycoprotein. IEF indicated the presence of all transferrin isoforms. No transferrin could be detected in the run-through fraction of the affinity chromatography.

Transferrin isoforms were separated by ion-exchange chromatography. Three fractions (F1, F2, and F3) were isolated in the serum of the healthy subject (Figure 1A). The protein content of each fraction was evaluated with
Analysis of N-linked glycans present on the serum transferrin isoforms of healthy subjects

N-linked glycans were enzymatically released from the transferrin isoforms by protein N-glycanase F (PNGase-F) digestion. The released oligosaccharides were purified by solid-phase extraction on porous graphitized carbon columns.

The released N-glycans from each transferrin isoform were first analyzed by fluorophore-assisted carbohydrate electrophoresis (Jackson, 1994). The electrophoretic profiles are shown in Figure 1B. The relative mobility of the 8-aminonaphthalene-1,3,6,-trisulfonate (ANTS) oligosaccharide fluorescent derivatives, expressed as the relative migration index (RMI) (Stack and Sullivan, 1992), was used as a measure of the association of a biantennary and a triantennary or from some tetrasialotransferrin having a 3/1 charge on the two oligosaccharides.

The released N-glycans from each transferrin isoform were further analyzed by mass spectrometry (MALDI MS). The mass spectrum only showed ions at m/z 2588 corresponding to an ANTS-derived oligosaccharide. The deduced monosaccharide composition corresponded to HexHexNAc2NeuAc2 (Klein et al., 1998). Interpretation of the electrophoretic profiles was completed with the RMI of the different ANTS-derived oligosaccharides (Stack and Sullivan, 1992; Hu, 1995).

The electrophoretic profile of ANTS-labeled oligosaccharides of fraction F3 contains a major band with a RMI of 6.7 and a broad band with a RMI between 8 and 9 that is absent of fractions F1 and F2 and corresponds to triantennary triasialylated oligosaccharides. Numerous minor bands are also observed with RMI ranging from 7 to 8. The oligosaccharide electrophoretic pattern of fraction F3, corresponding to penta- and hexasialotransferrins, is in agreement with the results of other groups and confirms that these isoforms are principally composed of the association either of a biantennary and a triantennary or of two triantennary fully sialylated oligosaccharides (de Jong et al., 1990; Landberg et al., 1995).

Fraction F2 is constituted principally of biantennary bisialylated oligosaccharides; fraction F1 mainly contains biantennary bisialylated oligosaccharides and two other bands having a slower mobility (RMI between 7.0 and 7.5) that probably correspond to monosialylated biantennary oligosaccharides or to monosialylated biantennary oligosaccharides (Stack and Sullivan, 1992). Nevertheless, all the observed bands could not be assigned to a precise oligosaccharide structure, and a mass spectrometric approach was performed to complete the analysis.

To avoid fragmentation by loss of terminal sialic acid, we used a method involving methylesterification of the carboxyl group, which allows the simultaneous analysis of neutral and sialylated oligosaccharides in the positive-ion detection mode (Powell and Harvey, 1996). As shown in Figure 2, the major ions at m/z 2274 present in all the studied fractions correspond to an oligosaccharide with a chemical composition of NeuAcMe2Hex5HexNAc4. The monosaccharide composition of each observed ion in the different fractions is summarized in Table I. The relative intensity of ions elucidates the combination of the two N-glycans present in each isoform. Fraction F1 contains mainly trisialotransferrin and, as shown by the two major ions seen in the mass spectrum, is the result of the association of a disialylated (m/z 2274) and a monosialylated (m/z 1969) biantennary oligosaccharide (Figure 2A). Minor ions at m/z 2420 correspond to a disialylated and fucosylated biantennary oligosaccharide. Fraction F2 contains mainly ions at m/z 2274, indicating that the association of two disialylated biantennary glycans constitutes the major part of tetrasialotransferrin. The distribution of negative charges due to the presence of sialic acids on the two oligosaccharides is 2/2. The minor ions at m/z 1969, corresponding to a monosialylated biantennary oligosaccharide, might have two different origins. It originates either from the presence of tetrasialotransferrin contaminating the fraction F2 (due to the limits of the resolution of the high-performance liquid chromatography column resulting in overlapping fractions) or from some tetrasialotransferrin having a 3/1 charge repartition on the two glycans. However the ions at m/z
2944 are weakly detected, suggesting a purification procedure origin.

The ions at 2639 correspond to a 365 mass increment due to an additional N-acetyllactosamine unit to the biantennary disialylated oligosaccharide (m/z 2274) (Table I).

Fraction F3 (Figure 2C) differs from the other by the presence of intense ions at m/z 2944 and 3090 corresponding to a triantennary trisialylated oligosaccharide, fucosylated or not. The repartition of the charge due to sialic acids on transferrin in fraction F3 is 2/3. Presence of ions at m/z 1969 might have two origins, either the association of a monosialylated N-glycan with a tetraantennary tetrasialylated oligosaccharide as indicated by the minor ions at m/z 3614 or the methylesterified sialic acids undergo a residual desialylation on the target during ionization in spite of the stabilizing effect of the chemical modification.

**Purification of transferrin isoforms from patients with severe alcohol abuse**

Human transferrin was isolated from the serum using the procedure described previously. Four fractions (F0, F1, F2, and F3) were obtained by ion-exchange chromatography (Figure 3A). IEF of the different fractions indicated that

<table>
<thead>
<tr>
<th>m/z</th>
<th>Chemical composition</th>
<th>Structure</th>
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<tbody>
<tr>
<td>1664</td>
<td>Hex₅HexNAc₄</td>
<td></td>
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<tr>
<td>1807</td>
<td>NeuAcMe Hex₅HexNAc₄</td>
<td></td>
</tr>
<tr>
<td>1869</td>
<td>NeuAcMe Hex₅HexNAc₄</td>
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</tr>
<tr>
<td>2274</td>
<td>NeuAcMe₂Hex₅HexNAc₅</td>
<td></td>
</tr>
<tr>
<td>2334</td>
<td>NeuAcMe₂Hex₅HexNAc₅</td>
<td></td>
</tr>
<tr>
<td>2420</td>
<td>NeuAcMe₂Hex₅HexNAc₅, DeoxyHex</td>
<td></td>
</tr>
<tr>
<td>2639</td>
<td>NeuAcMe₂Hex₅HexNAc₅</td>
<td></td>
</tr>
<tr>
<td>2944</td>
<td>NeuAcMe₂Hex₅HexNAc₅</td>
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<tr>
<td>3090</td>
<td>NeuAcMe₂Hex₅HexNAc₅</td>
<td></td>
</tr>
<tr>
<td>3614</td>
<td>NeuAcMe₂Hex₅HexNAc₅</td>
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The m/z values refer to [M + Na]⁺ ions.

Symbols for the structural formulae are defined as follows: closed square, GlcNAc; open square, Gal; open circle, Man; triangle, fuc; and open diamond, O-methyl ester of N-acetylneuraminic acid.
fraction F0 contains exclusively disialylated transferrin and that the electrophoretic profiles of the other fractions were similar to the corresponding fractions isolated from the control subjects.

**Analysis of N-linked glycans present on the serum transferrin isoforms of patients with severe alcohol abuse**

The oligosaccharides from each transferrin isoform from patients with severe alcohol abuse were first analyzed by fluorophore-assisted carbohydrate electrophoresis (Jackson, 1994). The electrophoretic profiles are shown in Figure 3B. Fractions F2 and F3 display a pattern similar to those observed in control subjects (Figure 1B). Fraction F0 appearing in patients with severe alcohol abuse has a profile similar to fraction F2 and indicates that most of the hypo-sialylated transferrin contains mainly bisialylated biantennary oligosaccharides and that disialotransferrin is the result of the loss of an entire oligosaccharidic chain.

The MS analysis indicates that the CDT fraction F0 principally contains the ions at \( m/z \) 2274 (Figure 4A), suggesting that this isoform is mainly originated from the absence of a complete oligosaccharidic chain. Nevertheless, a minor peak at \( m/z \) 1664 indicated the possible combination of two biantennary oligosaccharides with a charge repartition of 2/0. Finally, the ions at \( m/z \) 1969 indicate that a minor disialotransferrin glycoform is constituted with two monosialylated biantennary oligosaccharides with a charge repartition 1/1.

In fraction F1 isolated from patients with severe alcohol abuse, the presence of ions at \( m/z \) 2334 not observed in the normal subject (Figures 2A and 4B) indicates a monosaccharide composition of NeuAcMe\(_1\)Hex\(_6\)HexNAc\(_5\), suggesting two possible structures, either a biantennary or a triantennary oligosaccharide. To distinguish these two structures a digestion of fraction F1 with neuraminidase and/or galactosidase was performed.

In the digestion with both galactosidase and neuraminidase (Figure 5A), the two ions are found at \( m/z \) 1543 for the triantennary structure and at 1705 for the biantennary structure with a ratio of 10/1. In the digestion of methylsterified oligosaccharides with galactosidase (Figure 5B), ions at \( m/z \) 2010 (generated by the triantennary structure) and 2172 (generated by the biantennary structure) are detected in a similar ratio. From these experiments the original ions at \( m/z \) 2334 probably correspond to a mixture of a major compound, a triantennary monosialylated and a minor one, a biantennary monosialylated with an additional N-acetyllactosamine unit. These ions are also present in the tetrasialotransferrin fraction (F2).

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**Fig. 4.** MALDI-TOF MS analysis of carboxymethylated oligosaccharides released from the different isoforms of transferrin purified from the serum of a subject with severe alcohol abuse. (A) Carboxymethylated oligosaccharides isolated from fraction F0; (B) carboxymethylated oligosaccharides isolated from fraction F1; (C) carboxymethylated oligosaccharides isolated from fraction F2; (D) carboxymethylated oligosaccharides isolated from fraction F3. The \( m/z \) values of detected ions correspond to [M + Na\(^+\)] ions.

**Fig. 5.** MALDI-TOF MS analysis of exoglycosidase treatments of oligosaccharides isolated from fraction F1 from alcohol consumer transferrin. (A) Oligosaccharides of fraction F1 treated by neuraminidase and \( \beta1-4 \) galactosidase. (B) Methylsterified oligosaccharides of fraction F1 treated by \( \beta1-4 \) galactosidase. Symbols for the structural formulae are defined as follows: closed square, GlcNAc; open square, Gal; open circle, Man; triangle, fuc; and open diamond, O-methyl ester of N-acetylneuraminic acid.
Discussion

The aim of this work was to identify the different oligosaccharides present on the isoforms of transferrin purified from controls and from patients with severe alcohol abuse because the variation of their compositions might explain the mechanisms of the alteration of glycosylation observed in chronic alcohol intake. Oligosaccharides were isolated from each transferrin isoform and studied by MALDI MS and fluorescent carbohydrate electrophoresis. Ion-exchange chromatography or IEF detects the presence during chronic alcohol consumption of a hypiosialylated form of transferrin. In these assays, the hypiosialylated transferrin (CDT) is mostly constituted of the asialo- and the disialylated forms; in some methods a fraction of the trisialotransferrin is also taken into account. The results obtained in the present study indicate that the disialotransferrin present in the serum is principally the result of the loss of an entire N-linked oligosaccharide and that a minor fraction of the disialylated form is related to the loss of terminal sialic acids. There is a similarity between the glycosylation abnormality observed in patients with severe alcohol abuse and the glycosylation defect found in children suffering from congenital diseases of glycosylation type I (CDG type I), where the loss of a glycan is due to an abnormal biosynthesis and/or transfer of the dolichol-sugar intermediate. A decreased level of dolichol has been observed in rats fed ethanol (Cottalasso et al., 1996). Alcohol and products of ethanol oxidation (acetaldehyde) that can covalently bind to enzymes should alter the biosynthesis and/or transfer of the dolichol-oligosaccharide intermediates.

An interesting observation is the absence of a triantennary trisialylated oligosaccharide in the trisialotransferrin fraction (F1), suggesting the absence of a molecule constituted of a single oligosaccharide chain in this fraction. The lack of this glycoform of transferrin can be either an artifact of the analytical process, the chromatographic behavior on the MonoQ column of a trisialotransferrin constituted with a single oligosaccharide chain might be different from the other trisialylated isoforms, or the fact that during the processing of the oligosaccharide the N-acetyl glucosamine V transferase cannot function on a transferrin possessing a single chain.

The loss of an entire oligosaccharide chain is not the only modification of glycosylation observed during chronic alcohol intake. Alteration of terminal sialylation is demonstrated by the presence of (1) monosialylated N-glycan in the disialotransferrin fraction (F0); this structure also indicates that a minor fraction of CDT is a glycoprotein possessing two monosialylated biantennary N-glycans and (2) monosialylated triantennary oligosaccharide in the tri- and in the tetrasialotransferrin (F1 and F2).

In the mass spectra, the intensity of ions gives only a rough approximation of the relative quantities of each glycan. Nevertheless, the ratio of ions corresponding to monosialylated biantennary N-glycans (m/z 1969) present in tri- (fraction F1) and tetrasialylated transferrins (F2) appears similar when compared between controls and patients with severe alcohol abuse (compare mass spectra of Figure 2A with Figure 4B and Figure 2B with Figure 4C). Furthermore, there is not a major increase in the sera of the trisialotransferrin fraction (F1) relative to the tetra- and pentasialylated forms (F2 and F3).

In a recent observation, Dibbelt (2000) noticed that an increase of disialotransferrin was not accompanied by an increase of trisialotransferrin. This observation led the author to the conclusion that it is not the terminal sialylation of transferrin that is impaired in chronic alcohol intake (Dibbelt, 2000). Nevertheless, chronic alcohol intake has been shown to be responsible for alterations that should lead to an abnormal terminal sialylation. A decreased level of sialyltransferase activity has been observed during chronic alcohol consumption with a destabilisation of sialyltransferase mRNAs (Cottalasso et al., 1996; Lakshman et al., 1999; Rao and Lakshman, 1999). An increased activity of a hepatocyte membrane associated sialidase has also been described (Xin et al., 1995). As a result of these two mechanisms, an increase of transferrin oligosaccharides with terminal galactose residues should be observed. Furthermore, the catabolism of transferrin might influence the distribution of the different isoforms. Senescent glycoproteins, which have lost terminal sialic acids, are cleared from the serum by the hepatocytes; one of the pathways is mediated by the asialoglycoprotein receptor (Ashwell and Harford, 1982; Tozawa et al., 2001). Chronic alcohol intake impairs multiple aspects of the receptor mediated endocytosis (McVicker and Casey, 1999); the cellular redistribution and the altered biosynthesis of the asialoglycoprotein receptor (AGPR) have been demonstrated (Tworek et al., 1996). Modifications of the biosynthesis and of the catabolism of transferrin through chronic alcohol intake are complex and result from the sum of multiple and sometimes opposite effects. Monosialotransferrin is not detected by IEF or ion-exchange chromatography, disialotransferrin (F0) is mainly constituted of a transferrin with a single oligosaccharide fully sialylated, and the trisialotransferrin fraction (F1) is not increased during chronic alcohol intake. All these phenomena might be the result of the elimination rate of the hypoacetylated forms with terminal galactose residues by the hepatocytes, despite the partial alteration of the receptor-mediated endocytosis. Each glycovariant of transferrin has probably a different affinity toward the AGPR, and this affinity is dependent on the number of terminal galactose and sialic acid residues. A precise quantification of the terminal galactose residues in the most acidic transferrin might indicate the intensity of the phenomenon. Identification and quantification of the various alterations of the glycosylation are important to distinguish the markers of chronic alcohol consumption and of alcohol-related damages.

Furthermore, an interesting observation is that some patients with CDG type Ia signs and symptoms have a normal distribution of transferrin isoforms with decreased enzyme activities (Dupre et al., 2001). The relatively poor sensitivity of detection of chronic alcohol consumption by the measurement of CDT can probably be explained by genetic factors related to carbohydrate (Freeze, 2001) and ethanol metabolism (Yoshida, 1994).
Materials and methods

Materials

*Arthrobacter ureafaciens* neuraminidase (EC 3.2.1.18) and *Streptococcus pneumoniae* β-1-4 galactosidase (EC 3.2.1.23) were purchased from Glyco (Oxford, UK). Dowex AG 50 × 12 (H⁺) ion-exchange resin was from Bio-Rad (Marnes-la-Coquette, France). 2,5-Dihydroxybenzoic acid (DHB) was from Sigma-Aldrich (St-Quentin Fallavier, France). The mass calibration standard kit (calibration mixture 2) was purchased from Perseptive Biosystems (Framingham, MA).

Patient samples

Sera from patients with chronic alcohol abuse were collected in an alcohol-dependency clinic (Clinique de la Charité, CHRU de Lille). Control serum was obtained from volunteers.

IEF

Sera were saturated and diluted (1:20) with ferric citrate (40 μM) in the presence of sodium bicarbonate (50 mM) and separated on 1% agarose gels consisting of agarose IEF and ampholines with a range of 5.0–7.0 on a Phast-System (Amersham Biosciences, Orsay, France). The focused transferrin isoforms were visualized by immunofixation with an antibody coupled to activated Sepharose (Aminolink, Pierce, Rockford, IL) (Coddeville et al., 1985). Purification of transferrin was checked by SDS-PAGE using 7.5% acrylamide gels (Laemmli, 1970).

Purification of transferrin

Human serum (1.5–2 ml) was subjected to an affinity column made of rabbit anti-human transferrin IgG (Dako, Trappes, France) coupled to activated Sepharose (Aminolink, Pierce, Rockford, IL) (Coddeville et al., 1998). Purification of transferrin was checked by SDS-PAGE using 7.5% acrylamide gels (Laemmli, 1970).

Ion-exchange purification of transferrin glycovariants

The transferrin glycovariants were separated on a Mono Q HR 5/5 column (Amersham Biosciences) by a NaCl gradient in Bis-Tris buffer 20 mM, pH 6.2 (Jeppsson et al., 1993). Each fraction was collected, dialyzed against water, and lyophilized. Protein content of each fraction was evaluated with bicinchoninic acid (BCA protein assay reagent, Pierce, Rockford, IL) (Smith et al., 1985).

Release and purification of the transferrin glycans

Transferrin glycovariants (0.15–0.3 mg) were denatured at 100°C in the presence of SDS and β-mercaptoethanol. After addition of nonidet-P 40, PNGase-F (New England Biolabs, Herts, UK) was added (1 μl) (500,000 U/ml). After 2 h, 1 μl additional enzyme was added and left overnight.

The oligosaccharides were purified by solid phase-extraction on a graphitized carbon adsorbant (Alltech Associates, Templemars, France); the neutral and the sialylated oligosaccharides were eluted together with a solution of acetonitrile 25/water 75 containing trifluoroacetic acid (0.1%) (Packer et al., 1998).

Fluorescent carbohydrate electrophoresis

Oligosaccharides released by PNGase-F were labeled by reductive amination with ANTS (Molecular Probe, Leiden, Netherlands) using the protocol described by Powell and Harvey (1996). ANTS-labeled oligosaccharides were subjected to PAGE on an isocratic 30% polyacrylamide gel using the Tris-glycine discontinuous buffer (Laemmli, 1970), except that the SDS and the β-mercaptoethanol were omitted from all buffers. Images were acquired using a CCD camera.

Extraction and desalting of ANTS oligosaccharides from the gel

The excised gel piece was washed without shaking in 500 μl of water at 4°C for 30 min. The gel fragment was then removed and subsequently reincubated in 500 μl of water for 3–4 h at 4°C. During the second step, the derivatized glycans diffused out of the gel, and the efficiency of sugar extraction was directly checked using an UV light. The extracted oligosaccharides were lyophilized and further analyzed by MS.

Esterification of sialic acids.

To stabilize the sialic acid moiety under MALDI MS conditions, the sialic acid residues of PNGase-F-released oligosaccharides (500 pmoles) were stabilized by methylesterification of their carboxylic group (Powell and Harvey, 1996).

Exoglycosidase digestions.

Aliquots of methylesterified and unmodified N-glycans were subjected to exoglycosidase digestions onto the mass spectrometer target, as described by Colangelo and Orlando (1999). Briefly, 1 μl of oligosaccharide samples (50 pmoles) was incubated on the target with 800 U (0.25 μl) of neuraminidase or 56 U (0.25 μl) of β-1-4 galactosidase for 10 and 20 min with both enzymes. The enzymatic digestions were stopped by acidification with 1 μl of MS matrix.

MS. All mass spectra were acquired on a Voyager Elite (DE-STR) reflectron time-of-flight (TOF) mass spectrometer (Perseptive Biosystems) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. Samples were analyzed in delayed extraction mode using an accelerating voltage of 20 kV, a pulse delay time of 200 ns, and a grid voltage of 66%. Detector bias gating was used to reduce the ion current below masses of ~500 Da. After external calibration, between 100 and 200 scans were averaged for each of the reflectron mode spectra shown.

For all measurements, the dried droplet preparation technique was employed. Native and methylesterified N-glycans were cocrystallized with DHB as matrix (10 mg/ml DHB in an acetonitrile/water solution [70:30] containing 0.1% trifluoroacetic acid). ANTS-labeled oligosaccharides were mixed with a freshly made 3-aminoquinoline matrix solution (10 mg/ml of 3-aminoquinoline in methanol/water.
supplemented with 5 mM diammogorium hydroxide citrate). ANTS-labeled glycans were detected as [M-H]⁻ ions in linear negative mode, whereas the neutral sugars were observed as [M + Na]⁺ in positive reflectron mode.

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Abbreviations

AGPR, asialglycoprotein receptor; ANTS, 8-amino-naphthalene-1,3,6-trisulfonate; CCD, charged coupled device; CDG, congenital diseases of glycosylation; CDT, carbohydrate deficient transferrin; DHB, 2,5-dihydroxybenzoic acid; IEF, isoelectricfocusing; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PNGase F, protein N-glycanase F; RMI, relative migration index; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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