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Analysis of human transferrin glycopeptides by capillary electrophoresis and capillary liquid chromatography-mass spectrometry. Application to diagnosis of alcohol dependence

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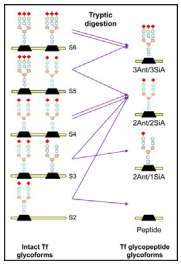
HIGHLIGHTS

- CE-TOF-MS and μLC-TOF-MS methods separate and detect Tf glycopeptide glycoforms.
- The surfactant used to enhance the enzymatic digestion interferes in CE analyses.
- Tf alteration in alcoholism is mainly due to entire loss of the carbohydrate chains.
- Ethanol intake has no effect in the activity of sialyltransferase and sialidase.
- This method could be used to elucidate Tf glycosylation in different subtypes of CDGs.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, capillary electrophoresis and capillary liquid chromatography coupled to mass spectrometry (CE-TOF-MS and μLC-TOF-MS) were used to detect and characterise human transferrin (Tf) glycopeptide glycoforms obtained by tryptic digestion. After selecting μLC-TOF-MS because of improved performance in analysis of N_{413} and N_{611} glycopeptide glycoforms, the proposed methodology was applied to serum samples. Two immunoaffinity columns were employed to isolate Tf from serum samples. Both columns were activated with the same anti-Tf antibody but using two different bonding chemistries. After immunoaffinity purification and digestion, serum samples from a teetotal individual (as control) and from individuals with low and high alcohol dependence were analysed by μLC-TOF-MS. Relative abundance of each glycoform was useful to estimate the degree of alcohol dependence of each individual. Finally, the established methodology was used to analyse serum samples from specific individuals with an unknown degree of alcohol dependence.

1. Introduction

Human transferrin (Tf) is an iron-binding serum glycoprotein of ~80 kDa, the main function of which is the transport of iron through the blood plasma [1]. Approximately 5.8% of its total molecular mass corresponds to one O-glycan with one hexose unit at serine 32, and two complex type N-glycans attached to asparagines 413 and 611 of the polypeptide backbone, respectively. Consequently, owing to the microheterogeneity associated with complex type N-glycans, Tf exists as a mixture of glycoforms which differ in composition, structure and charge. The main glycoform of Tf is tetrasialoform (S4) and it presents two disialylated biantennary glycans (globally, it shows 4 antennae with one terminal sialic acid each (SiA = N-acetylneuraminic acid)). The less abundant

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glycoforms in a healthy individual comprise disialoform (S2) with a disialylated biantennary glycan (2Ant/2SiA), trisialoform (S3) with a disialylated and a monosialylated biantennary glycan (2Ant/2SiA and 2Ant/1SiA), pentasialoform (S5) with one biantennary and one triantennary glycan (2Ant/2SiA and 3Ant/3SiA) and hexasialoform (S6) with two fully sialylated triantennary glycans (3Ant/3SiA). S1 and S0 glycoforms, with less than 2 sialic acid residues, are barely existent in a healthy individual [2–4]. These unusual glycoforms are known as carbohydrate deficient transferrin (CDT) and they have been proposed as biomarkers for the diagnosis of different diseases such as chronic alcohol dependence or congenital disorders of glycosylation (CDG) [2–4].

Chronic alcohol dependence represents a serious human health risk that usually ends in premature death due to different causes, such as hepatic cirrhosis, internal haemorrhage, alcohol poisoning, hepatocarcinoma, accidents or suicide. Whereas the mechanism that alters Tf glycoform composition in CDG is quite well understood [5], the process that causes abnormality in Tf glycosylation in cases of heavy alcohol consumption is still a matter of debate. On the one hand, some authors have suggested that ethanol intake may alter the biosynthesis and/or transfer of dolichol-oligosaccharide intermediates, impeding initiation of N-linked glycosylation and thus causing the loss of the entire carbohydrate chain, as happens in CDG type I [6,7]. On the other hand, other authors have proposed that ethanol also decreases the activity of sialyltransferase and stimulates sialidase activity, which may result in partial N-linked oligosaccharide or sialic acid loss [8,9].

Capillary electrophoresis (CE) and anion-exchange liquid chromatography (LC) with UV detection are the current techniques employed for the analysis of CDT glycoforms [4]. However, UV detection does not permit unequivocal identification of Tf glycoforms [10]. Moreover, these methodologies are insufficient to fully clarify the mechanism behind transferrin carbohydrate abnormality, not only in cases of heavy alcohol consumption but also in some CDG subtypes. Nowadays, mass spectrometry (MS) is the most reliable way to obtain structural information about protein glycosylation [11,12]. The analysis of Tf glycoforms by MS can be approached either by analysing the intact glycoprotein [10] or by analysing the glycans or glycopeptides obtained by enzymatic digestion [13,14]. However, the first approach is rather challenging due to difficulties in detecting intact glycoproteins with sufficient sensitivity by MS [15,16]. The detection of specific glycosylation markers of lower molecular mass (glycopeptides or glycans) could be a better alternative for detecting CDT glycoforms by MS. In this context, most authors have studied Tf glycoforms through analysis of released N-glycans. However, in contrast to glycans, glycopeptides not only provide information about the structure and composition of the oligosaccharides, but also about glycosylation sites and their degree of occupancy. Some authors have studied CDT glycoforms by MALDI-MS; however, under typical vacuum source conditions, MALDI may result in dissociation of labile glycosidic bonds in glycan and glycoconjugate analytes [3,13,17–20]. Because it can be tuned to be considerably softer than MALDI, ESI seems to be more appropriate for profiling intact native glycans or glycopeptides without dissociation of labile sugars such as sialic acid and fucose.

In the present study, capillary electrophoresis and capillary liquid chromatography coupled to electrospray time-of-flight mass spectrometry (CE-TOF-MS and μLC-TOF-MS) were evaluated for the analysis of human Tf glycopeptides. Due to the complexity of serum samples, prior to enzymatic digestion and analysis by μLC-TOF-MS, an off-line immunoaffinity chromatography purification of the glycoprotein was performed using an anti-Tf polyclonal antibody. After desalting and tryptic digestion, the resulting digests were analysed by μLC-TOF-MS, obtaining a glycopeptide glycoform map of Tf with which teetotallers could be distinguished from individuals with different degrees of alcohol dependency.

2. Materials and methods

2.1. Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Isopropanol (iPrOH), acetic acid (HAc, glacial), formic acid (HFor, 98–100%), trifluoroacetic acid (TFA, ≥99%), glycine (≥99.7%), ammonia (25%), ammonium acetate (NH_4Ac) and sodium hydroxide were supplied by Merck (Darmstadt, Germany). CNBr-activated Sepharose 4B was from GE Healthcare (Waukesha, WI, USA). DL-Dithiothreitol (DTT, ≥99%), iodoacetamide (IAA), ammonium hydrogen carbonate, water (LC-MS grade) and acetonitrile (LC–MS grade) were supplied by Sigma–Aldrich (Madrid, Spain). Ethylenediaminetetraacetic acid (EDTA, ≥99%) was supplied by Panreac (Barcelona, Spain). Trypsin (sequencing grade modified) was provided by Promega (Madison, WI, USA). RapiGest® from Waters (Bedford, MA, USA) was used to enhance enzymatic digestion. Goat polyclonal antibody against Tf (immunogen affinity purified) was purchased from Abcam (Cambridge, UK). ESI low concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for tuning and calibration of the oa-TOF mass spectrometer.

2.2. Tf samples

Tf standard was purchased from Sigma–Aldrich (Madrid, Spain). Tf standards were prepared at 1500 mg L^{-1} in 50 mM NH_4HCO_3 buffer (pH 7.9).

Serum samples with a non-pathological profile (SERODOS® plus) and serum control samples from individuals with high and low alcohol dependence were purchased from Human GmbH (Wiesbaden, Germany) and Bio-Rad (Hercules, CA, USA), respectively. Two serum samples from individuals with an unknown degree of alcohol dependence were provided by Balagué Center S.A. (Barcelona, Spain).

Tf standards as well as serum samples previously purified by immunoaffinity chromatography were reduced, alkylated and immediately subjected to enzymatic digestion. Briefly, 3.75 μL of 0.5 M DTT in 50 mM NH_4HCO_3 buffer was added to an aliquot of 100 μL of Tf 1500 mg L^{-1} with 0.1% (w/v) RapiGest®. The mixture was incubated in a TS-100 Thermo-Shaker digester (Bio-San, Riga, Latvia) at 56 °C for 30 min and then alkylated with 50 mM IAA for 30 min at room temperature in the dark (10.5 μL of 0.73 M IAA). Excess reagent was removed by ultracentrifugation with Microcon YM-10 (MW cut-off 10 kDa, Millipore, Bedford, MA, USA), washing 3 times with NH_4HCO_3 buffer [21,22]. The final residue was recovered from the upper reservoir by being centrifuged upside down in a new vial, and reconstituted in 100 μL NH_4HCO_3 buffer with 0.1% RapiGest®. Trypsin in an enzyme to sample ratio of 1:40 by mass was added and the mixture was carefully vortexed and incubated at 37 °C in the digester for 18 h. At the end of digestion, RapiGest® was hydrolysed to avoid MS interferences. TFA was added to a concentration of 0.5% (v/v) and the mixture was incubated in the digester at 37 °C for 45 min. Then, the solution was centrifuged for 10 min at 12,000 rpm to separate the RapiGest® residues. The supernatant was carefully collected and stored at −20 °C until analysis. pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain). Centrifugation procedures were carried out in a Mikro 20 centrifuge (Hettich, Tuttlingen, Germany) at room temperature.

2.3. Immunoaffinity chromatography (IAC)

2.3.1. Preparation of IAC columns

2.3.1.1. Silica-hydrazide column. The silica-hydrazide column was prepared as reported elsewhere [23]. Briefly, 0.05 g of silica-diol