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A capillary zone electrophoresis method for detection of Apolipoprotein C-III glycoforms and other related artifactually modified species☆

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ABSTRACT

ApolipoproteinC-III (ApoC-III) is a human plasma glycoprotein whose O-glycosylation can be altered as a result of congenital disorders of glycosylation (CDG). ApoC-III exhibits three major glycoforms whose relative quantification is of utmost importance for the diagnosis of CDG patients. Considering the very close structures of these glycoforms and their tendency to adsorb on the capillary, a thorough optimization of capillary electrophoresis (CE) parameters including preconditioning and in-between rinsing procedures was required to efficiently separate all the ApoC-III glycoforms. Permanent coatings did not contribute to high resolution separations. A fast and reliable method based on a bare-silica capillary combining the effect of urea and diamine additives allowed to separate up to six different ApoC-III forms. We demonstrated by a combination of MALDI-TOF mass spectrometry (MS) analyses and CE of intact and neuraminidase-treated samples that this method well resolved glycoforms differing not only by their sialylation degree but also by carbamylation state, an undesired chemical modification of primary amines. This method allowed to demonstrate the carbamylation of ApoC-III glycoforms for the first time. Our CZE method proved robust and accurate with excellent intermediate precision regarding migration times (RSDs < 0.7%) while RSDs for peak areas were less than 5%. Finally, the quality of three distinct batches of commercial ApoC-III obtained from different suppliers was assessed and compared. Quite similar but highly structurally heterogeneous ApoC-III profiles were observed for these samples.
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1. Introduction

Glycosylation is one of the most important post-translational modifications of mammalian proteins. It corresponds to the attachment of an oligosaccharidic chain to the protein backbone by a complex enzymatic process [1]. There are two types of glycosylation, N- and O-glycosylation depending on the nature of the amino acid residue linked to the oligosaccharidic chain. Both N- and O-glycosylations can be affected in congenital disorders of glycosylation (CDGs). CDGs are inherited metabolic diseases caused by mutations of any gene coding for enzymes implied in the glycan biosynthesis [2]. CDGs affecting N-glycosylation can be defined as type I or type II according to the defective biosynthesis step [3]. The type I corresponds to a variable glycosylation site occupancy while type II is defined by the alteration of the carried N-glycan motifs. In addition to disorders associated with defects in the N-glycosylation machinery, O-glycosylation pathways can also be affected. The detection of ApolipoproteinC-III (ApoC-III), an O-glycosylated plasma protein was first proposed by Wopereis et al. [4], as a potential biological test complementary to that of transferrin to monitor abnormal N-glycosylation [5], to screen rather abnormal O-glycan structures [4] occurring as a consequence of

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mutations in genes encoding for conserved oligomeric Golgi complex [6–8].

ApoC-III is a 79 amino acid protein synthesized by the liver and the intestine. It is a component of circulating particles in blood rich in triglycerides and is mainly present in very-low density lipoproteins [9] while its plasmatic concentration is comprised between 0.1 mg mL^{-1} and 0.5 mg mL^{-1} in plasma [10,11]. ApoC-III carries the most common form of O-linked glycans, a mucin type core 1 at threonine 74 which corresponds to a galactose β1-3 linked to an N-acetylgalactosamine residue. Three main glycoforms have been described [12–14], depending on the number of sialic acid residues, *i.e.* from 0 to 2 (ApoC-III$_0$, ApoC-III$_1$ and ApoC-III$_2$ forms, respectively). Recently, minor ApoC-III glycoforms containing high levels of fucosylation instead of sialylation have also been reported [15,16] (fucosylated glycans have Lewis-type structures which are different from the mucin type core 1 type of sialylated glycans).

Several analytical techniques have been developed to analyze ApoC-III in plasma for diagnostic purposes. Electrophoresis-based methods, such as IEF [4,12,17,18] and two-dimensional electrophoresis [19–23] can be successfully used for glycoform analysis and allowed to separate the two sialylated glycoforms from the asialylated one. However, such techniques are only semi-quantitative and quite time consuming. Besides, new developments in intact ApoC-III analysis by matrix assisted laser desorption ionization (MALDI) coupled to either time of flight (TOF) [19,21,23–27] or Fourier transform ion cyclotron resonance [16] have been reported. MALDI techniques enabled the relative quantitation of glycoform ratios (ApoC-III$_1$/ApoC-III$_0$ and ApoC-III$_2$/ApoC-III$_0$) but also a differentiation, in human plasma, of the ApoC-III$_0$ into two main isoforms, one without any glycan and the second bearing one asialoglycan [21,27]. The determination of those ratios allows the diagnosis of O-glycosylation related CDGs. Moreover, the sample preparation was simple as only plasma purification/delipidation and desalting steps using solid-phase extraction method were necessary. The coupling of high-performance liquid chromatography to MS using an internal standard from NeoBioSci (ApoC-III deuterated on 3 alanine residues) allowed the evaluation of absolute abundances of all glycoforms [28] by performing targeted multiple-reaction monitoring detection.

Based on its automation, speed and ease of use, capillary electrophoresis (CE) is an alternative and quantitative technique that deserves to be considered for the analysis of glycoproteins. To the best of our knowledge, no CE method has yet been developed for the analysis of glycosylated ApoC-III in biological fluids. The main objective of the study was to implement a CZE method for the quantitative monitoring of ApoC-III glycoforms in plasma of CDG patients. In that aim, a well characterized and quantified standard solution of ApoC-III had to be defined. We therefore purchased ApoC-III (purified from human plasma) from different providers, but the first CZE analyses revealed a higher structural heterogeneity than expected (*i.e.* not limited to O-glycosylation). This has prompted us to refocus our objective on the thorough characterization and separation of the various protein forms from three distinct batches of commercial ApoC-III obtained from different suppliers. The present paper reports the numerous efforts made in that direction.

2. Materials and methods

2.1. Reagents and consumables

ApolipoproteinC-III (95%) provided as solution (1 mg mL^{-1} in 10 mM ammonium bicarbonate pH 7.4) was obtained from Sigma Aldrich (reference A3106, St. Louis, MO, USA), from Merck (reference ALP60, Darmstadt, Germany) and from Antibodies online GmbH (reference ABIN491549, Aachen, Germany). All first experiments were conducted with ApoC-III from Sigma Aldrich.

Acetonitrile, boric acid (99.5%), 1,4 diaminobutane (DAB, 99%), dimethyl sulfoxide (DMSO, 99%), neuraminidase type VIII from Clostridium perfringens (85%), sinapinic acid (99%), sodium dodecyl sulfate (SDS, 98.5%), trifluoroacetic acid (TFA), trypsin from bovine pancreas (TPCK treated) and urea (99%) were all obtained from Sigma Aldrich. Sodium phosphatemonobasic (NaH_2PO_4, 99.1%) and di-sodium phosphate dibasic (Na_2HPO_4, 99.8%) were purchased from Thermo Fisher scientific (Waltham, MA, USA). Sodium hydroxide (1 M), hydrochloric (1 M) and acetic (99.9%) acids were obtained from VWR (Fontenay-sous-Bois, France).

All buffers were prepared using deionized water and were filtered through a 0.22 μm nylon membrane (VWR) before use. Deionized water was prepared with a Direct-QR 3 Water Purification System from Millipore (Billerica, MA, USA).

Bare fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA), polyvinylalcohol (PVA) and polyacrylamide (PAA) coated capillaries from Agilent Technologies (Santa Clara, CA, USA) and Sciex (Framingham, MA, USA), respectively.

2.2. Neuraminidase digestion

Neuraminidase digestion of ApoC-III was performed to remove α 2–3, α 2–6 and α 2–8 linked sialic acids as follows. Firstly, 10 units of lyophilized neuraminidase was dissolved in 300 μL of 50 mM sodium phosphate buffer pH 6.0. Then 20 μL of the standard ApoC-III at 1 mg mL^{-1} in the sample buffer (10 mM ammonium bicarbonate pH 7.4) were mixed, by pipetting, with 2 μL of the neuraminidase solution (0.066 units for 20 μg of protein). The digestion was statically performed overnight at 37 °C in an oven.

2.3. Trypsin digestion

ApoC-III was first 2-fold diluted in 100 mM ammonium bicarbonate pH 7.9 (50 μL of ApoC-III at 1 mg mL^{-1} mixed with 50 μL of 100 mM ammonium bicarbonate). Trypsin digestion was performed upon the addition of 2 μL of trypsin solution at 0.5 mg mL^{-1} (enzyme:protein ratio (w/w) of 1:50). The digestion was statically performed overnight at 37 °C in an oven and was then stopped by the addition of 2 μL of formic acid.

2.4. MALDI-TOF MS analysis

MALDI-TOF MS analysis of intact ApoC-III was performed essentially as described before [21,27], but with minor modifications. Briefly, analyses were performed on a Bruker Ultraflextreme MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam-II laser. Each ApoC-III sample (0.5 μL at 1 mg mL^{-1}) was spotted on the MALDI target and thoroughly mixed on-target with 0.5 μL of matrix composed of a saturated solution of sinapinic acid in 50:50:0.1, Water/ACN/TFA. MS spectra of intact ApoC-III were acquired at 2 kHz laser repetition rate in the positive linear ion mode, with a 20 kV acceleration voltage and an extraction delay of 250 ns. All spectra were obtained by accumulating ∼1000 laser shots over the 5000–20,000 *m/z* range.

MALDI-TOF MS of tryptic peptides were acquired, using α-cyano-4-hydroxycinnamic acid as matrix (10 mg mL^{-1} in 50% ACN containing 0.1% TFA), at 2 kHz laser repetition rate in the positive reflectron ion mode, with a 20 kV acceleration voltage and an extraction delay of 130 ns. All spectra were obtained by accumulating ∼1000 laser shots over the 500–5000 *m/z* range. Confirmation of peptide structures can be obtained from MS/MS spectra acquired in LIFT mode, at 1 kHz laser repetition rate applying 7.5 kV for initial

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