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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.

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

acceleration of ions and 29.5 kV for reacceleration of fragments in the LIFT cell.

2.5. Capillary electrophoresis analysis

CE experiments were carried out with a P/ACE ™ MDQ instrument (Sciex) equipped with a diode array detector. UV detection was performed at 214 nm. Bare fused silica capillaries with an internal diameter of 50 μm and an effective length to the detector of 50 cm (total length of 60.2 cm) were used. Software 32 Karat™ version 7.0 (Beckman Coulter, High Wycombe, UK), was used to control the instrument and to collect data. The final background electrolyte (BGE) was 150 mM sodium borate pH 9.5, containing 4 M urea and 1 mM DAB. It was prepared as follows: a stock solution of 0.5 M boric acid was prepared by dissolving 1.546 g of boric acid in 50 mL of water. To prepare the BGE, 21.2 mL of the stock solution of boric acid were mixed with 7.5 mL of 1 M NaOH. Then, 12.012 g of urea were added to the buffer which was heated at 30 °C to facilitate urea dissolution. It was then followed by the addition of 5.1 μL of DAB (99%). The buffer pH was checked and adjusted with 1 M NaOH. Deionized water was added to a total volume of 50 mL. This BGE was stored at 4 °C for not more than two weeks. Fused silica capillaries were preconditioned by flushing them at 20 psi with MeOH for 10min, H_2O for 10 min, 1 M HCl for 10 min, H_2O for 10 min, 1 M NaOH for 10 min, H_2O for 10 min and BGE for 10 min. Next, the capillary was filled with 0.1 M NaOH and stored during 3 h. After this storage, the capillary was rinsed and equilibrated with the BGE for 10 min and then 20 kV was applied during 3 h. The capillary was emptied and rinsed with fresh BGE for 10 min before starting an analysis. Each BGE vial was renewed after each analysis and each sample was analyzed in triplicate. A plug of sample was hydrodynamically injected from the inlet end by applying a pressure of 0.5 psi for 6 s. The separation was carried out under 20 kV at 25 °C. After each run, the capillary was rinsed for 5 min with 25 mM SDS solution and 5 min with H_2O, and equilibrated with the BGE for 10 min. At the end of the day, the capillary was rinsed for 5 min with 25 mM SDS and 10 min with H_2O and dried with air before storage at room temperature. All conditions were carefully optimized as described in this article.

2.6. Fractionation of ApoC-III by CZE and control of fractions by MALDI-TOF MS

A large plug of sample (5% of the capillary volume) was injected from the inlet end by applying a pressure of 1.5 psi for 17 s. A first separation step was carried out under 20 kV at 25 °C for 15 min. Then the outlet BGE vial was removed and replaced by a vial containing 5 μL of the sample buffer. A second separation step was then performed under the same conditions as the first one but during 0.5 min for the collection of the peak of interest.

3. Results and discussion

The objective of this work was to develop a CZE method for separating and quantifying the different ApoC-III glycoforms. Method implementation and optimization were first conducted with commercially available plasma-derived ApoC-III. Thus, we paid particular attention to limit protein adsorption to the capillary and also to electrophoretically resolve glycoforms differing from each other by only one sialic acid residue. Classical parameters such as the pH, the ionic strength of the BGE, the nature of the capillary coating or the addition of additives to the BGE were thoroughly examined. Also, rinsing and preconditioning procedures were carefully optimized to improve the method robustness.

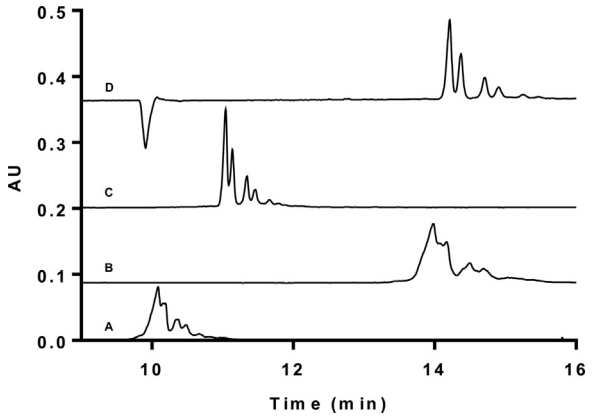

Fig. 1. Comparison of CZE profiles obtained for ApoC-III without additive in the BGE (A), and using different additives in the BGE as 1 mM DAB (B), 4 M urea (C) or a combination of 1 mM Dab and 4 M urea (D).
Sample: 1 mg mL^{-1} ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate pH 9.5, separation conditions: capillary: bare fused silica, 50 μm, 50 cm (effective length), voltage: normal polarity, 20 kV, temperature: 25 °C, injection: 0.5 psi for 6 s.

3.1. Optimization of the CZE method for the ApoC-III glycoform separation

3.1.1. Optimization of the running buffer

As proteins are prone to adsorption on fused silica capillary and in order to develop a method compatible with mass-spectrometry detection, we have chosen first to work with permanent capillary coatings. We tested two commercial neutral coatings, PVA and PAA. As the isoelectric point (p*I*) of ApoC-III is comprised between 4.5 and 5.0, and no peak was detected at pH 5.0 with both coatings. At pH 7.0 peaks were observed for both PAA and PVA (Fig. S1) but they were very broad (around 2 min) precluding convenient resolution improvements.

We then decided to work without coatings but using repelling conditions (pH > pI) in bare fused silica capillary and therefore under counter EOF mode. Under this configuration, high resolution is expected if the electrophoretic mobility (μep) of the analytes is closed to that of the electroomostic flow (μeo). We optimized the pH (from 7.0–10), the ionic strength (from 50 to 150 mM) and the composition (sodium phosphate, sodium borate and sodium tetraborate) of BGE. Using sodium borate buffer, as soon as the ionic strength was increased, a splitting of each peak into two peaks was observed. The beneficial effect of borate can be explained by a selective complexation between borate and cis hydroxyl groups of the glycan moieties [29]. The best separation conditions were obtained with 150 mM sodium borate buffer pH 9.5. Surprisingly, we observed six distinct peaks gathered into three pairs of peaks for the commercial ApoC-III sample (Fig. 1A), while only three corresponding to the different glycoforms were expected. Many efforts were made first to separate those species as much as possible and then to better understand the origin and nature of these peaks (*vide infra*).

To increase the electrophoretic resolution, different types of additives were added to the running buffer. Firstly, the addition of diaminobutane was tested at different concentrations (from 1 mM to 10 mM). It appeared that 1 mM DAB allowed increasing the resolution between each group of peaks (Fig. 1B) while the resolution between the first two peaks was still insufficient (Rs = 0.5). DAB has been reported to prevent protein adsorption, by interacting with the negatively charged silanols groups that leads also to a decrease of the μeo [30]. This resolution improvement was obtained thanks to the reduced μeo intensity becoming closer to the μep of ApoC-III glycoforms. However, this induced an increase of migration times

Table 1
Impact of the inter run rinsing and preconditioning procedures on the repeatability of the method (n = 3).

Capillary Preconditionning procedure	Inter run Rinsing procedures	RSD (%) of migration times	RSD (%) of relative peak areas
Classical[a]	0.1 M NaOH	0.3–0.5	27–38
Classical	25 mM SDS	1.2–1.3	3.2–11.7
Classical	25 mM SDS-0.1M NaOH	4.5–7.0	19–27
Classical + voltage[b]	25 mM SDS	1.4–3.0	9.3–10.7
Classical + storage 0.1M NaOH[c] + voltage	25 mM SDS	0.26–0.32	0.3–3.2

Sample: 1 mg mL^{-1} ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1 mM DAB, 4 M urea pH 9.5.
[a] Classical preconditioning: MeOH (10 min), H_2O (10 min), 1 M HCl (10 min), H_2O (10 min), 1 M NaOH (10 min), H_2O (10 min) and BGE (10 min).
[b] Voltage step: capillary filled with BGE and under 20 kV during 3 h.
[c] Storage step: capillary stored with 0.1 M NaOH during 3h.

and peak width. Concentrations over 3 mM DAB were detrimental to the resolution due to a too low μeo.

Secondly, urea was added to the separation buffer (150 mM sodium borate pH 9.5 without DAB) at different concentrations (from 2 M to 6 M). Sharper peaks and a much higher resolution within each group of peaks and also between the first two peaks (Rs = 0.8) were observed (Fig. 1C) with 4 M of urea. Urea is a chaotropic agent that disrupts hydrogen bonds [30], resulting in protein unfolding which yields higher differences between charge-to-hydrodynamic volume ratios of the coexisting glycoforms in the case of ApoC-III. No noticeable effect on the resolution was observed using concentration above 4 M.

The profile obtained with a borate buffer pH 9.5 containing 1 mM DAB and 4 M urea showed the synergic effect of both additives with three well resolved pairs of peaks (Fig. 1D). Moreover, the resolution between the first two peaks was greatly improved (Rs = 1.3). This condition was selected for all further experiments with the ApoC-III glycoforms.

Finally, modifications of other parameters such as capillary length or voltage were also investigated but did not lead to significant improvements (data not shown).

3.1.2. Optimization of the rinsing and preconditioning procedures

First experiments were performed with a classical inter-run 0.1 M NaOH rinsing step and demonstrated an excellent intra-day repeatability of migration times (tm) (RSD < 0.5%) but a less acceptable one for the peak areas (A) (RSD around 30%). In this study, BGE was prepared freshly using urea powder and not a urea stock solution, so urea decomposition was most likely not an issue and some residual adsorption of ApoC-III onto the capillary wall, leading to a certain lack of repeatability, was rather suspected. With the aim to improve the repeatability, different rinsing procedures involving more stringent conditions were compared. It appeared that among all tested procedures, the rinsing with 25 mM SDS allowed to achieve the most satisfactory RSDs but always insufficient for A (<1.3% for tm and 12% for A) (Table 1).

We also carefully optimized the preconditioning procedure. Each new capillary was initially conditioned with a rather classical procedure involving different successive washing steps as described in Section 2.6. We suspected that the DAB layer was not enough stable and homogenous to ensure sufficiently repeatable A. Thereby, we investigated the addition of one equilibration step with the running buffer under a 20 kV voltage application during 3 h after the classical preconditioning. This step was expected to create a more homogenous DAB coating of the capillary *via* competitive interaction between DAB [31] and impurities from the buffer with the free silanols [32]. Such equilibration step was previously used in particular for the CZE separation of the recombinant human erythropoietin (rhEPO) in the monography of the European Pharmacopeia [33]. In our case, it slightly improved the repeatability of the A (RSD < 10.7%), but at the expense of the tm one (Table 1). We then tried to include, between the common preconditioning and the equilibration step under a 20 kV voltage, another additional equilibration step under capillary storage in 0.1 M NaOH during 3 h. This step is supposed to ensure the complete ionization of surface silanol groups in order to promote a full coating of silanol groups by DAB. The combination of these two additional steps led to an excellent repeatability for both tm (RSD < 0.4%) and A (RSD < 3.3%), rendering the method robust and reproducible. Finally, the inter-day (n = 3) repeatability of the method was evaluated. An excellent intermediate precision was obtained for tm (RSD < 0.7%) and a satisfactory one for A (RSD < 5%). A stability study of capillary preconditioning was also performed. After 16 consecutive analyses of commercial standard ApoC-III sample (Fig. S2) repeatability of tm was excellent (RSD < 0.9%) and the resolution of the less resolved peaks (peaks 1 and 2) was stable (RSD = 6.2%). Over one month of use and 40 injections of different ApoC-III samples, we evaluated the repeatability of the capillary preconditioning using two analyses performed at the beginning and two at the end of this study. The capillary was still highly stable with a satisfactory repeatability of tm (RSD < 2.0%) corresponding to an excellent repeatability of the μep (RSD ≤ 1.0%) and the resolution between peaks 1 and 2 was always stable (RSD = 4.8%). Concerning the repeatability of different batches of capillaries (n = 3), the repeatability of migration time was acceptable (RSD < 1.5%) and it corresponds to an excellent repeatability of the μep (RSD < 0.5%). The repeatability of relative areas ($rA = A/\Sigma Ai$) of the four main peaks was acceptable too (RSD < 5%) and the resolution between peaks 1 and 2 was still very stable (RSD = 4.8%). All those results show the high stability of the method.

3.2. Analysis and characterization of ApoC-III by CZE-UV and MALDI-TOF mass spectrometry

3.2.1. Analysis of intact and desialylated ApoC-III

With the final optimized conditions, the CZE glycoform pattern of commercial ApoC-III isolated from plasma, unexpectedly showed three pairs of peaks (Fig. 2A) instead of three expected single peaks.

In order to clarify the identity of these different species, the ApoC-III sample was treated with neuraminidase to remove terminal sialic acids from the O-glycan chain (Fig. 2B). Sialylated glycoforms exhibit more negative charges than their asialylated counterpart, leading to a decrease of their apparent mobility. Migration times of desialylated ApoC-III were consequently decreased. Upon neuraminidase treatment, we expected a single peak corresponding to the asialylated ApoC-III but instead we observed three distinct peaks, potentially highlighting the presence of additional post-translationally or chemically modified protein species. To investigate further the nature of this modification, MALDI-TOF MS analysis of the intact and desialylated ApoC-III samples was performed. Under these conditions, three main clusters of peaks differing by 43 Da mass increments were observed for the intact ApoC-III sample and corresponded to various ApoC-III isoforms (Fig. 3 A). ApoC—III_0 (*m/z* 9131), ApoC—III_1 (*m/z* 9422) and ApoC—III_2 (*m/z* 9713) glycoforms bear 0–2 sialic acid residues, respectively. The most intense peak (*m/z* 9508) in Fig. 3A presumably corresponds to the monosialylated ApoC-III with two 43Da

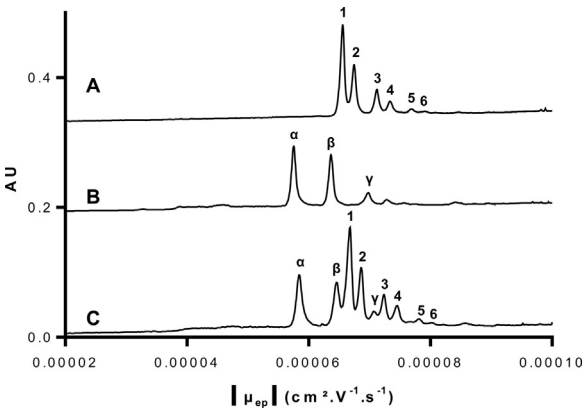

Fig. 2. CZE profiles of ApoC-III before (A) and after (B) enzymatic desialylation with neuraminidase and (C) a mix of both samples.
Sample: 1 mg mL^{-1} ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1 mM DAB, 4 M urea pH 9.5, separation conditions as in Fig. 1. Peaks α, β and γ correspond to carbamylated species and peaks 1–6 correspond to both sialylated and carbamylated species.

mass increments. Such mass differences are consistent with the presence of several carbamyl groups on ApoC-III. Carbamylation is an *in vitro* nonenzymatic chemical modification of peptidic chains where an isocyanate group is covalently linked to the ε-amino groups of lysine residues and to the N-terminal residues of proteins/peptides [34]. This modification can be artificially introduced during sample preparation with urea (through urea degradation) [35] mimicking *in vivo* carbamylation. In aqueous solutions, urea can readily dissociate upon heating or after prolonged incubation or storage to form isocyanate and ammonium ions. We suspected that such an artifactual carbamylation could occur during the purification and extraction process of ApoC-III from human plasma. *In-vitro* carbamylation of ApoC-III during an ion exchange chromatographic step using high concentration of urea has been already reported by Herbert et al. [36]. Since a similar purification method was used by the ApoC-III provider (Sigma) [37], one cannot exclude the occurrence of carbamylation under these conditions. Undesired protein carbamylation can hamper the process of protein analysis, especially the tryptic digestion step since carbamylated lysine will not be cleaved anymore by trypsin. Of note and as previously described by others [21,25], the ApoC—III$_0$ glycoform (*m/z* 9131) and related species observed in the MALDI mass spectrum might be viewed as artifactually formed by sialic acid loss, phenomenon particularly favored at high laser power [25]. This asialylated-glycoform is almost never detected in 2D electrophoresis [21], and is therefore probably present at only very low levels *in-vivo* [25]. The artefactual loss of sialic acid residues observed under our MALDI conditions might also explain why the relative abundances of species bearing one or two 43 Da mass increments slightly fluctuate between mono- and di-sialylated protein species (Fig. 3A). Another potential explanation would be that the di-sialylated ApoC-III can be carbamylated to a lesser extent due to the additional steric hindrance resulting from the presence of a second sialic acid residue. Also, it might be linked to an artefactual loss of carbamyl moiety under our MALDI conditions, since such modification also seem quite labile and is rapidly lost under MS/MS conditions (vide infra). Nicolardi et al. [38], reported the detection of up to six minor multifucosylated ApoC-III glycoforms in some serum samples. With the exception of a particular trifucosylated species, these fucosylated glycoforms were only detected is less than 20% of the 96 serum samples tested, which may explain why none of such structures was detected in our MALDI-TOF experiments performed on specific commercial ApoC-III samples.

After neuraminidase treatment, the MALDI-TOF MS spectrum (Fig. 3B) showed a cluster of four peaks corresponding presumably to the asialo-form ApoC—III$_0$ (*m/z* 9131) still differing by successive mass increments of 43 Da. Only three among these four species were detected by CZE-UV, the three major peaks, α, β and γ, observed in Fig. 2B corresponding potentially to ApoC-III species bearing 1–3 carbamyl groups. One explanation could be the poor detectability of the minor native ApoC—III$_0$ by UV (Fig. 2B). The disappearance of peaks above 9400Da upon neuraminidase treatment further confirms the sialylated nature of ApoC-III glycoforms initially detected by MALDI-TOFMS (Fig. 3).

3.2.2. *Identification of carbamylated amino acid residues*

In order to confirm the occurrence of carbamylation and identify the modified amino acid residues, a tryptic digestion of the intact ApoC-III sample was performed and the resulting peptides analyzed by MALDI-TOF and MALDI-TOF/TOF. From these experiments, we first confirmed the presence of 43 Da mass differences between certain pairs of peaks observed in the MALDI-TOF mass spectrum of the tryptic digest of ApoC-III, thus signing potential car-

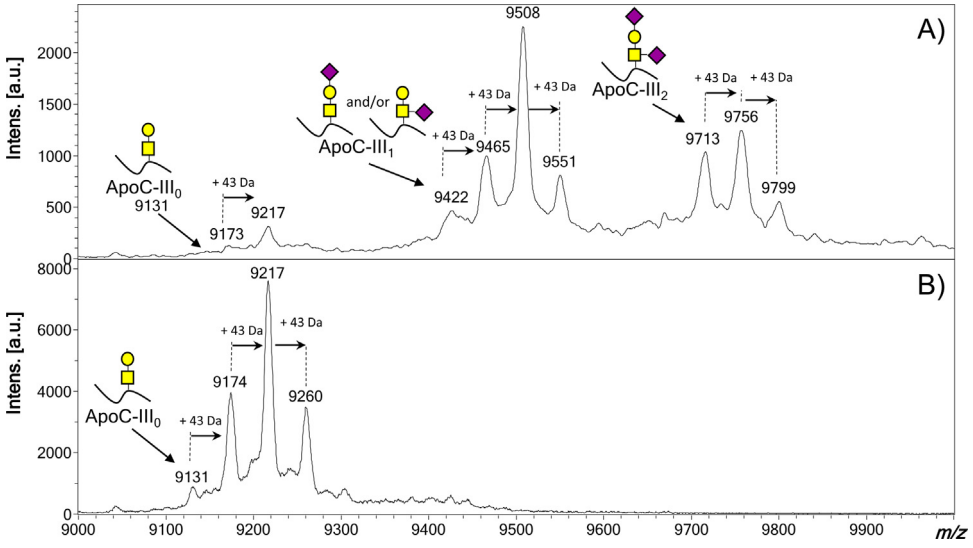

Fig. 3. MALDI-TOF MS profile of the intact ApoC-III (A) and desialylated ApoC-III sample (B). Sample: 1 mg mL^{-1} ApoC-III in 10 mM ammonium bicarbonate pH 7.4, matrix: saturated solution of sinapinic acid in 50:50:0.1 water/ACN/TFA, positive linear mode, accumulation of 1000 laser shots, range: 5000–20000 Da.

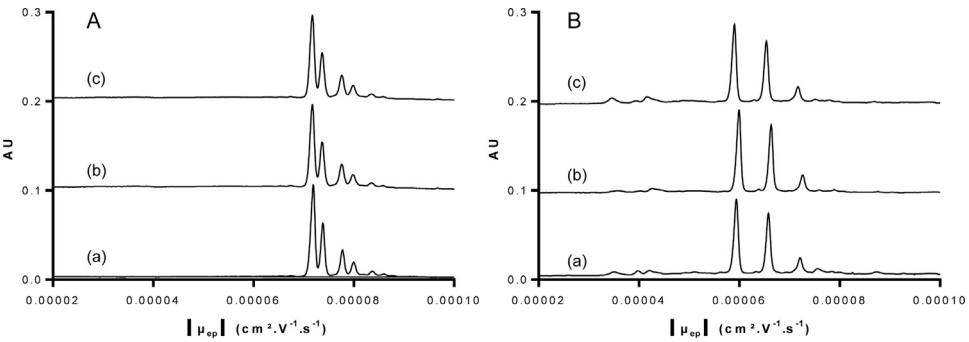

Fig. 4. Comparison of the three samples of intact ApoC-III by CZE before (A) and after (B) neuraminidase treatment; (a) sample from Merck, (b) sample from Antibodies online, (c) sample from Sigma Aldrich.
Sample: 1 mg mL^{-1} ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1 mM DAB, 4 M urea pH 9.5, separation conditions as in Fig. 1.

bamylated peptides. In addition, the MALDI-TOF/TOF mass spectra of the corresponding species are characterized by the presence of neutral losses of CHNO (−43 Da), a specific feature of carbamylated peptides (Fig. S3) [39,40]. Of note, carbamylation precludes efficient trypsin digestion at modified lysine residues, giving peptides with a miscleavage site. From the MALDI-TOF/TOF tandem mass spectra and by comparing carbamylated/native peptide ratios, we deduced that the modification preferentially occurred on the N-terminus of ApoC-III and on Lys51, and to a lesser extent on Lys17 or Lys24. Fig. S3 shows the MALDI-TOF/TOF spectra of the two mostly (as judged from MALDI-TOF mass spectra) carbamylated peptides (N-terminus and Lys51). Due to its lower pKa value (∼8.0), the N-terminus of the protein is presumably more prone to carbamylation than the ε-amino-groups of lysines [36]. The preferential carbamylation of some lysine residues might be related to the three-dimensional structure of ApoC-III, resulting in a favored solvent accessibility of these particular sites.

3.2.3. *Tentative identification of the peaks observed by CZE-UV*

Based on these results, the three peaks obtained in the CZE profile of the desialylated sample (Fig. 2B) were preliminarily attributed to three different carbamylated states (1C to 3C) of ApoC-III$_0$ glycoform. As expected, the asialylated-glycoform ApoC-III$_{0a}$ existing in the low concentration range *in-vivo* [38] was not detected. It appeared that the differences of electrophoretic mobilities between peaks α/β and β/γ were identical (6.12×10^{-6} cm^2 V^{-1} s^{-1} with a difference between both values lower than 0.1%). As desialylation was performed and proved efficient (vide supra), the species detected and represented on Fig. 2B only represent the heterogeneity arising from carbamylation. Therefore, these differences of electrophoretic mobilities likely account for the successive additions of one carbamyl moiety.

As mentioned above, carbamylation corresponds to the addition of an isocyanate moiety to a primary amino group of a peptidic chain, and can thereby result in an increase of both hydrodynamic volume and the overall negative charge of the protein of interest (*e.g.* by neutralizing the positive charge carried by lysine residues). Both factors influence the apparent electrophoretic mobility in opposite sense and this modification might result in a decrease of glycoform apparent electrophoretic mobilities. Thus, glycoforms with low levels of carbamylation should migrate first while highly carbamylated species should present lower apparent electrophoretic mobilities and appear later under our conditions. Therefore, the peak α observed in Fig. 2B might correspond to the isoform with one carbamyl moiety added while peaks β and γ would result from the addition of two and three carbamyl moieties respectively.

Moreover, this N-terminal amino-group has a pKa around 8.0 and thus does not carry a positive charge at pH 9.5 (separation pH) so carbamylation should not affect its charge [36]. Thus, this N-terminal carbamylated form should have the same electrophoretic mobility than the non-carbamylated form leading to a co-migration of both forms. Thereby, this co-migration of the mono and non-carbamylated forms could be another explanation for the detection of only three peaks in the CZE profile of the desialylated ApoC-III. This could also potentially explain the difference of the relative abundances of each carbamylation state between CZE and MALDI-TOF profiles. Complementary experiments proved that the high concentrations of urea present in the BGE did not induce any carbamylation of ApoC-III, which is also consistent with previous reports on 2D gel electrophoresis of carbamylated proteins [41].

In a separate set of experiments, we collected some fractions from CZE runs of the intact sample and analyzed them by both CZE and MALDI-TOF MS (Fig S4). Although tending to confirm preliminary peak attributions (vide supra), this experiment did not bring much information since collected peaks were not pure enough (Fig. S4) Moreover, MALDI-TOF analysis revealed that the collection introduced an additional level of structural heterogeneity with the formation of oxidized species (+16 Da). ApoC-III contains two methionine residues that can become readily oxidized [42,43]. Altogether, these drawbacks limit the usefulness of such fractionation experiments.

In Fig. 2A, we observed three groups of doublet peaks. It seems that the sialylation has led to the duplication of the peaks observed in Fig. 2B and thus peaks 1 and 2 differ only from a sialic acid residue. In order to compare more accurately desialylated and sialylated samples, we analyzed a mixture of both samples (Fig. 2C). Under these conditions, we expected but did not observe similar μep differences between peaks differing by one sialic acid residue, *e.g.* between peaks α/1 and 1/2. Thereby, it appeared that it becomes difficult to anticipate the migration of sialylated and carbamylated species. Indeed, one additional level of complexity might come from negatively charged sialic acids present in those glycoforms that could have strong intramolecular electrostatic interactions with primary amines of the amino acids chain or with DAB present in the BGE. Such phenomenon could drastically impact the μep of sialylated species and could explain why the μep differences observed in the carbamylated sample are different from those obtained in carbamylated and sialylated samples.

Altogether these data underline that it is really difficult to correctly assign peaks one by one as long as ApoC-III is both silalylated and carbamylated. This would require further complex and time consuming complementary experiments, which are not in the scope of this article. However, the identification of differentially sialylated species can be easier to address by working on a non-carbamylated (*i.e.* native) ApoC-III sample.

3.3. Comparison of ApoC-III batches from different suppliers

The optimized CZE method was then used to analyze different batches of human plasma-derived ApoC-III obtained from three different providers to see if the presence of carbamylated is a widespread phenomenon. All batches were analyzed both by MALDI-TOF MS (Fig S5) and CZE (Fig. 4A) to assess their proteoform profile, their purity being the same according to the suppliers (95%). It appeared that all commercial ApoC-III samples contained both carbamylated and sialylated forms of ApoC-III to a very similar extent. Indeed, based on both MALDI-TOF MS and CZE profiles, all three samples were qualitatively and quantitatively homogenous, which suggests that a similar ApoC-III purification protocol was used by the different suppliers. Complementary analyses of samples obtained after desialylation further confirm this observation (Fig. 4B). It should be noted that Trenchevska et al. [26] used a commercial ApoC-III sample coming from RayBiotech and did not encounter such modified glycoforms. The CZE method developed here allowed therefore to separate isoforms depending on their carbamylation degree. To the best of our knowledge, this work is the first one reporting an artefactual carbamylation of commercial samples of ApoC-III. Some *in-vitro* carbamylated proteins, such as green fluorescent protein [44] or human serum-albumin (HSA) [45], have already been monitored by capillary IEF or CZE respectively. HSA carbamylation has been demonstrated responsible for the shift in the albumin peak symmetry which could be used to assess their carbamylation degree. However, quantification of carbamylated products could not be performed due to a too low resolution [45].

4. Conclusion

In this work, we developed for the first time a fast and accurate CZE method for the separation of intact ApoC-III isoforms. The resolution of the separation was very satisfactory thanks to the use of an alkaline sodium borate buffer combined with the synergic effect of urea and DAB. CZE separation of sialylated ApoC-III glycoforms and their related carbamylated species proved successful even if the presence of modified commercially available ApoC-III standard drastically hamper the development of a quantitative CZE method. An optimization of the preconditioning and rinsing steps contributed to make the method robust. MALDI-TOF MS experiments combined with CZE of intact and neuraminidase-treated samples enabled to demonstrate for the first time the carbamylation of ApoC-III. The present method can also be used as a quality control tool for evaluating batch-to-batch consistency of plasma-derived as well as recombinantly expressed ApoC-III.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2017.12.002

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