Comparison between chromatofocusing and icIEF charge variant profiles of unconjugated monoclonal antibodies and their drug conjugates

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□ ABSTRACT □

Assessing charge variant profiles is one of the tools used to insure the quality of monoclonal antibody (mAb) and their drug conjugates (ADC). In this study, the charge variant profiles obtained by weak anion exchange chromatofocusing and icIEF methods for two unconjugated antibodies (mAb-1 and mAb-2) and their maytansine conjugates were compared. Significant differences were observed between chromatofocusing and icIEF profiles for studied mAbs and ADCs. The charge chromatofocusing profile of mAb-1 showed two main peaks (pI values 8.1 and 8.2) and one minor peak (pI value: 7.9). While its icIEF profile demonstrated two main peaks (pI values: 7.9 and 8.1) and two minor peaks (pI values: 7.7 and 8.2). mAb-2 was more basic and homogenous with one major peak (pI value 8.7) using chromatofocusing method and two peaks (pI values: 8.9 and 9.0) using icIEF method. The studied conjugated antibodies had more heterogeneous and basic charge variant profile than their unconjugated mAbs. The charge variant profile for studied ADCs varied between chromatofocusing and icIEF methods. icIEF method was capable to separate the charge variants of mAbs and their conjugates with good resolution in comparison to the chromatofocusing method and therefore was more powerful to characterize and to determine the presence of any changes in charge variant profile of these products.

Keywords: Antibody, conjugated, charge isoforms, pI, icIEF, chromatofocusing.

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مقارنة بين مرتسمات الكروماتوغرافيا البؤرية وicIEF لمغايرات الشحنة لأضداد وحيدة النسيلة عارية ومرتبطة مع دواء

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ملخّص

يعد مراقبة مرتسم مغايرات الشحنة أحد الأدوات المستخدمة لضمان جودة الأجسام المضادة وحيدة النسيلة (mAb) العارية او المرتبطة مع دواء (ADC). في هذه الدراسة، تمت مقارنة مرتسمات مغايرات الشحنة التي تم الحصول عليها باستخدام الكروماتوغرافيا البؤرية المعتمدة على استخدام أعمدة مبادلة ضعيفة للأيونات السالبة مع المرتسمات التي تم الحصول عليها باستخدام الرحلان الكهربائي البؤري الشعري المصور icIEF لاثنين من الأضداد (mAb-1, mAb-2) سواء كانت عارية أم مرتبطة مع دواء المايتانسين. وجدت فروق هامة بين مرتسمات الكروماتوغرافيا البؤرية ومرتسمات icIEF لمغايرات الشحنة للأضداد العارية والمرتبطة. أظهر مرتسم مغايرات الشحنة باستخدام الكروماتوغرافيا البؤرية لـ mAb-1 قمتين رئيستين(pI: 8.1 و8.2) وقمة واحدة ثانوية (pI: 7.9). في حين أن مرتسم مغايرات الشحنة باستخدام icIEF لهذا الضد أعطى قمتين رئيستين (pI: 7.9 و8.1) وقمتين ثانويتين (pI: 8.2, 7.7). أظهر mAb-2 تجانساً وقلوية أعلى من mAb-1 مع قمة رئيسية (pI 8.7) باستخدام الكروماتوغرافيا البؤرية وقمتين (pI: 8.9 و9.0) باستخدام icIEF. عند ربط الأضداد الى دواء سام للخلايا ازداد عدم تجانس وقلوية مرتسمات مغايرات الشحنة مقارنة مع الأضداد الموافقة. اختلفت مرتسمات مغايرات الشحنة للأضداد المرتبطة بين طريقة icIEF وطريقة الكروماتوغرافية البؤرية. اثبتت طريقة icIEF قدرة أكبر على فصل مغايرات الشحنة سواء لاضداد عارية عارٍ او مرتبطة مع دواء مع معامل فصل جيد مقارنة مع الطريقة الكروماتوغرافية البؤرية وبالتالي كانت أكثر ملاءمة لوصف واظهار أي تغيرات في مرتسم مغايرات الشحنة لهذه المنتجات.

الكلمات المفتاحية: الأضداد، الأضداد المرتبطة، مغايرات الشحنات، نقطة التعادل الكهربائي، الكروماتوغرافيا البؤرية، icIEF.

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Introduction:

Therapeutic monoclonal antibodies (mAbs) are a very growing class for targeted treatment of different diseases, essentially cancer [1-2]. Nearly half of more than 100 mAbs approved by the US Food and Drug Administration (FDA) were anticancer [3]. Many types of mAbs are used as antitumor (unconjugated mAbs, mAb fragments, or antibody derivatives) [4]. The most common used type in the treatment of cancer is the unconjugated mAbs. Conjugating small toxic molecules to mAbs by cleavable or non-cleavable linker can improve their antitumor efficiency, leading to a new type (antibody-drug conjugates ADCs) [5-12]. ADCs can selectively deliver cytotoxic drugs to targeted cancer cells, leading to their apoptosis.

Through the development of mAbs or ADCs, full characterization is required to ensure their stability, product purity from batch to batch, the pathways of degradation, etc [13-16]. Various modifications in the protein structure of antibody, such as glycosylation, aggregation, oxidation, or deamidation, may lead to a considerable heterogeneity of mAb and ADC size and charge. Furthermore, linking several drug molecules per antibody decreases the homogeneity of ADCs [15-16]. These probable modifications may lead to the presence of different related species in crude and final mAb or ADC products.

One of the main quality attributes of mAb or ADC characterization is the determination of charge variants [17]. Modifications of pI may modify the pharmacokinetics of mAb or ADCs and therefore their biological activity [18].

Gel isoelectric focusing electrophoresis (IEF) and capillary isoelectric focusing electrophoresis (cIEF) are used to determine the charge variant profile of mAbs and ADCs according to their isoelectric point (pI) [19]. cIEF offers several advantages compared to traditional IEF such as good reproducibility, high resolution, no needs to dyes for detection, automation and speed and quantitative analysis [20]. In the conventional cIEF, the focalization of analytes is followed by subsequent mobilization of the focused sample zone to the detection point by different methods. Imaged cIEF (icIEF) overcame the mobilization step where the detection occurs along the entire length of a column (whole-column detection WCD) [21].

Another method used to characterize the charge variant profile of mAbs and ADCs is anion and cation chromatofocusing methods [22-27]. This methods involve the separation of proteins based on their isoelectric point (pI) by generating a gradient of pH through ion exchange stationary phases [28-29].

The different separation mechanisms between icIEF and chromatofocusing techniques may be lead to a difference of charge variant profile of mAbs and ADCs. This study aimed to characterize the charge isoform profiles of two unconjugated monoclonal antibodies (mAb-1 and mAb-2) and their conjugates to a cytotoxic maytansine derivative by a weak anion exchange chromatofocusing method and compared the obtained profiles with those from icIEF method.

Materials and methods

Reagents:

The used reagents were: diethanolamine, urea, sucrose, histidine and glycine (Sigma-Aldrich), hydrochloric acid (VWR), Pharmalyte solutions (pH 3-10 and 8-10.5) (GE Healthcare), polybuffer 96 (GE Healthcare), NaCl (Acros). Kit ICE280 chemical test, Kit iCE280 electrolytic solution, methyl cellulose 1%, 0.5%, and pI Markers (6.6, 8.18 and

9.5) were purchased from Convergent Bioscience. Salt and buffer solutions were prepared in deionized water.

Protein samples:

Two monoclonal naked antibodies and their maytansine conjugates were analysed. The studied antibodies were formulated in HGS buffer consisted of histidine 10mM, glycine 130mM, and Sucrose 5% (w/v).

Sample preparation for analysis by icIEF

A protein sample was prepared by diluting to a desired final concentration in 0.35% methyl cellulose, 4% pharmalytes (3–10) and pharmalytes (8–10.5) (1:1 ratio), 2M urea and pI markers (6.61, 8.18, 7.05, 9.5) were added to the sample for pI calibration. Each test sample was then vortexed by centrifugation. After centrifugation, the sample was transferred to a glass autosampler vial and centrifuged to remove bubbles before placing in the autosampler carousel for analysis.

Chromatofocusing apparatus

All experiments were performed on Dionex ICS-3000 chromatographic instrument controlled by Chromeleon® software (version 6.80) obtained from Dionex (Sunnyvale, CA, USA). The used column was Mono P 5/200 GL (particle size: 10 µm, 200x5mm I.D) (GE Healthcare). The chromatographic conditions were: flow rate 1ml/min, λ: 280nm, and injection quantity: 200 or 500µg. The mobile phase composed of; (A): start buffer (0.025M diethanolamine-hydrochloric acid pH 10.5), (B): elution buffer (polybuffer 96-hydrochloric acid 8%, 8-10.5 pharmalytes 0.2%, pH 6.0) and (C): washing phase (2M NaCl). The applied elution gradient was presented in figure 1. As there is no pI markers in this method, pH of the fractions collected was measured in order to estimate the pI of charge isoforms.

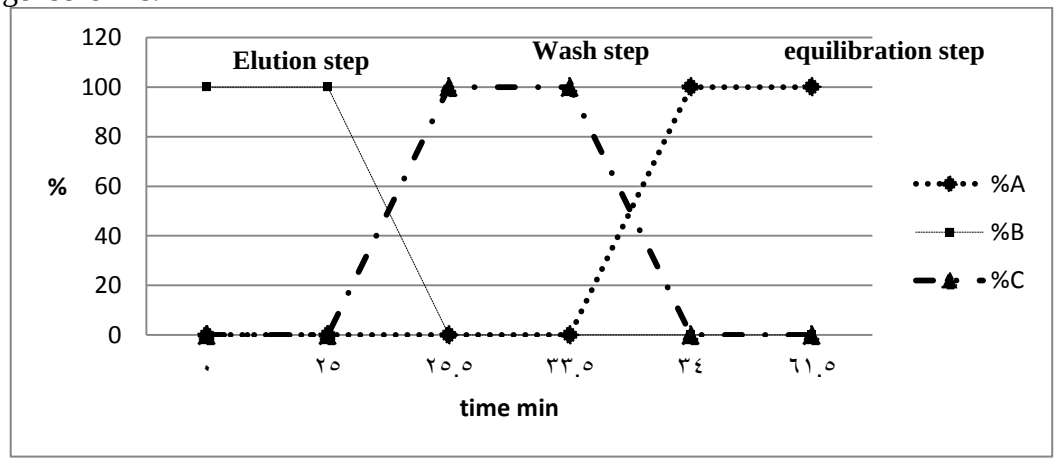


Figure 1: The gradient of ; (A): start buffer (0.025M diethanolamine-hydrochloric acid pH 10.5), (B): elution buffer (polybuffer 96-hydrochloric acid 8%, 8-10.5 pharmalytes 0.2%, pH 6.0) and (C): washing phase (2M NaCl).

icIEF apparatus

The icIEF analysis was conducted using an iCE280 instrument with PrinCE autosampler and capillary cartridge from Convergent Bioscience. A transparent capillary column (50mm, 100µm ID, 200µm OD) is embedded into the glass cartridge with its inner surface coated with a fluorocarbon to minimize electroosmotic flow. Reservoirs for cathodic (100mM NaOH, 0.1% methyl cellulose) and anodic solutions (80mM H3PO4, 0.1% methyl cellulose) were attached to the glass cartridge and separated from the capillary by the hollow fiber membrane. Protein focusing time was 10 or 12min at 3000V and detection at 280nm was achieved with a charge-coupled device (CCD) camera.

Results and discussion

Chemical changes of mAbs after translation, which refer to post-translational modifications (PTMs), increase their heterogeneity and lead to the formation of acidic and basic isoforms beside the main specie [30]. Monitoring the charge variants of mAbs is required to ensure their consistent profile during the development phases and in the final products. To determine the charge variant profile of mAb, analytical methods capable to separate and quantify the charge species should be used. The aim of this study was to compare the charge heterogeneity profiles obtained by two methods (anion exchange chromatofocusing and icIEF) for two monoclonal antibodies (mAb-1 and mAb-2) and their drug conjugates.

Comparison of chromatofocusing and icIEF profiles of the unconjugated mAbs

mAb-1 is an anti-CD19 monoclonal antibody designed to target the cell surface antigen CD19, found on a number of B-cell-derived cancers [31]. mAb -2 is anti-EphA2 where EphA2 receptor is one of 16 related receptor tyrosine kinases (RTKs) that are activated by membrane-associated ligands known as ephrins. EphA2 protein levels have been reported to be elevated in many types of cancer [32].

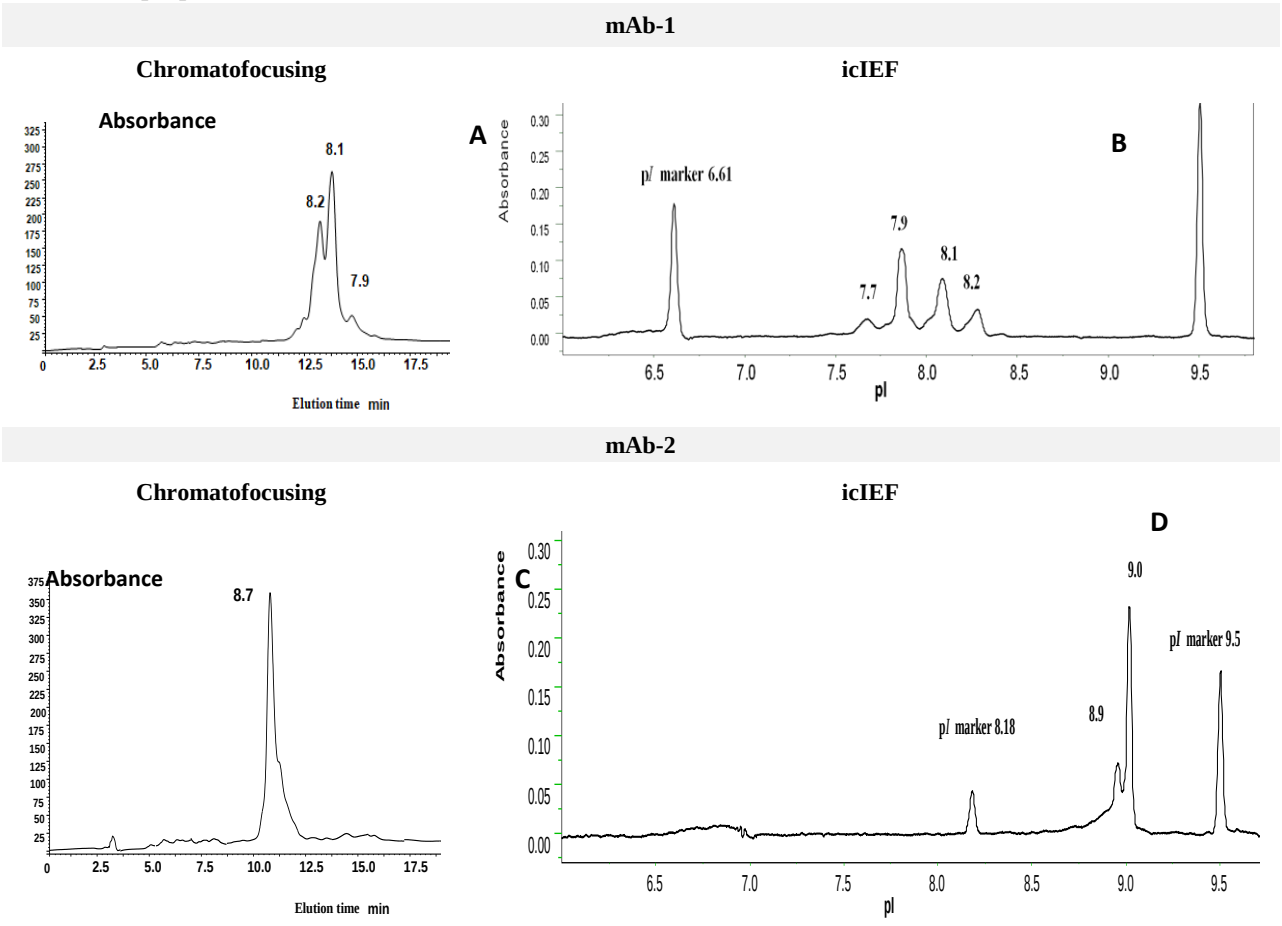


Figure 2: Chromatofocusing profile of naked mAb-1 (A) and mAb-2 (C): experimental conditions was as mentioned in material and methods, Injected quantity: 200µg. pI values: mAb-1 (8.1 and 8.2), mAb-2 (8.7).
icIEF profile of unconjugated mAb-1 (B) and mAb-2 (D). Final concentration of unconjugated antibody in sample matrix is 0.2mg/ml diluted in 0.35% methyl cellulose, 4% 3–10 pharmalytes/ 8– 10.5 pharmalytes (1:1 ratio), 2M urea. pI markers: 8. 18, 9.50. Focusing time :10min at 3000V.

Charge variant profiles of studied unconjugated mAbs obtained by chromatofocusing and icIEF methods were presented in Figure 2. A good separation of charge variants were obtained by the icIEF method in comparison to the chromatofocusing method for both unconjugated mAb-1 and mAb-2. mAb-2 showed more homogeneous and basic charge variant profile than that for mAb-1.

Significant differences were observed between chromatofocusing and icIEF profiles for both mAb-1 and mAb-2. The % area for charge variants of unconjugated mAb-1 notably varied between the icIEF and the chromatofocusing methods (Figure 3). With chromatofocusing method, mAb-1 had two main charge isoforms (pI values: 8.1 and 8.2) and one minor charge isoform (pI value: 7.9). With icIEF, two main charge isoforms (pI values: 7.9 and 8.1) and two minor charge isoforms (pI values: 7.7 and 8.2) were observed for mAb-1. The % area of peak corresponding to pI value 7.9 increased from 2% (chromatofocusing) to 43% (icIEF). In contrast, the % areas of peaks corresponding to pI values 8.2 and 8.1 decreased from 46% and 56% (chromatofocusing) to 34% and 15% (icIEF) respectively.

The icIEF charge variant profile for mAb-2 was more basic (two charge isoforms with pI values 8.9 and 9.0) than the chromatofocusing one (one major charge isoform with pI value 8.7).

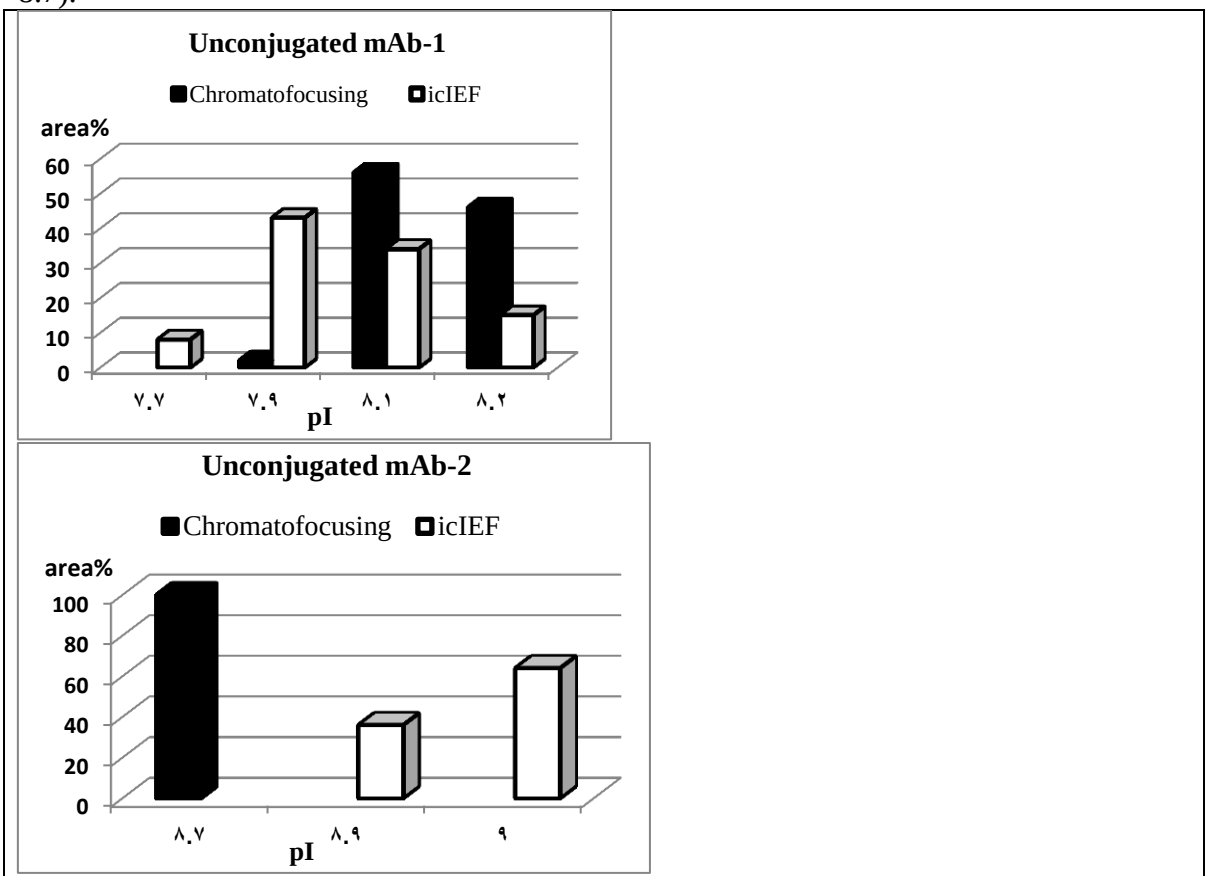


Figure 3: Comparison of % area of unconjugated mAb-1 and mAb-2 charge isoforms obtained by two methods (anion exchange chromatofocusing and icIEF)

Comparison of chromatofocusing and icIEF profiles of conjugated mAb-1

Because of the systemic toxicity of the natural product "maytansine", targeted approaches were investigated to selectively deliver this agent to cancer cells, among them monoclonal antibodies.

mAb-1 was conjugated to a derivative of the cytotoxic agent maytansine by an optimized cleavable linker. The ratio of the cytotoxic agent is around 3.5 moles per mole of the antibody. Figure 4 demonstrated the chromatofocusing and the icIEF profiles for the conjugated mAb-1. The conjugated mAb-1 was more heterogeneous than corresponding unconjugated antibody. This heterogeneity of conjugated mAb-1 is related to the covalently linking of cytotoxic drug to the free amine groups of lysine of mAb. mAbs often contain 40-60 lysine residues. Increasing the number of lysine amine groups conjugated to a linker molecule leads to a decrease in pI of ADC (more acidic) [33-35]. Baylon *et al.* conducted a study for charge variant characterization of IgG1-Fc and conjugated IgG1-Fc [35]. The study showed that the chemical conjugation of IgG1-Fc to different drugs via the amino acid of mAb surface Lys led to a decrease in pI upon conjugation.

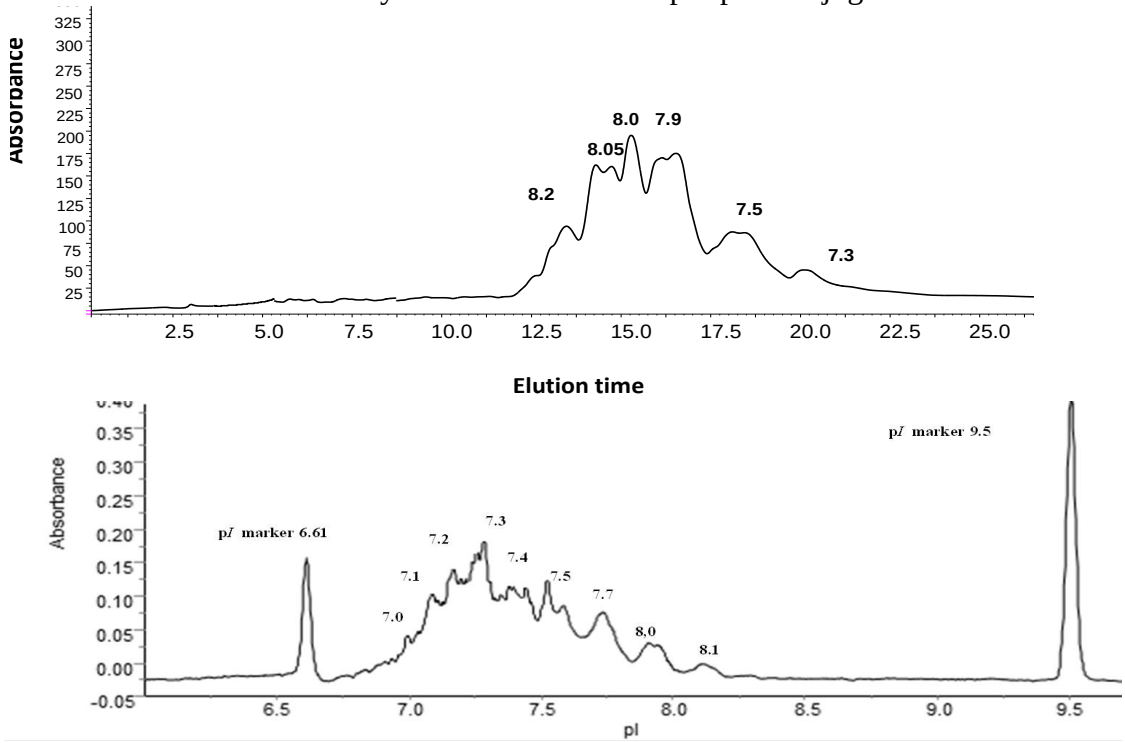


Figure 4: Chromatofocusing profile of conjugated mAb-1 (A): experimental conditions was as mentioned in material and methods, Injected quantity: 500µg. pI values: 8.2, 8.05, 8.0, 7.9, 7.5 and 7.3. icIEF profile of of conjugated mAb-1 (B). Final concentration of unconjugated antibody in sample matrix is 1mg/ml diluted in 0.35% methyl cellulose, 4% 3–10 pharmalytes/ 8– 10.5 pharmalytes (1:1 ratio), 2M urea. pI markers: 8. 18, 9.50. Focusing time :12min at 3000V.

The icIEF profile of the conjugated mAb-1 was more heterogeneous (ΔpI:1.1, pI range: 7.0–8.1) than its chromatofocusing profile (ΔpI: 0.9, pI range: 7.3-8.2). icIEF method was capable to separate the charge variants of conjugated mAb-1with good resolution.

The % area of charge variants obtained by the icIEF method were compared with those obtained by the chromatofocusing method (Figure 5). Charge variants of pI values 7.9, 8.5 and 8.2 were only observed by chromatofocusing, whereas charge variants of pI values 7.0, 7.1, 7.2 and 8.2 were only observed by icIEF. The level of unconjugated antibody in an

ADC formulation is a critical parameter in process control because it can directly affect the efficacy of ADC. Using chromatofocusing method, 12% of mAb1-drug conjugate had a pI of 8.2. Using icIEF method, 15% and 6% of mAb1-drug conjugate had pI values 7.7 and 8.1. These pI values revealed the presence of the unconjugated mAb in the ADC sample. Higher level of unconjugated antibody in ADC sample was demonstrated by icIEF profile (21%) compared to that observed in the chromatofocusing profile of conjugated mAb-1 (12%).

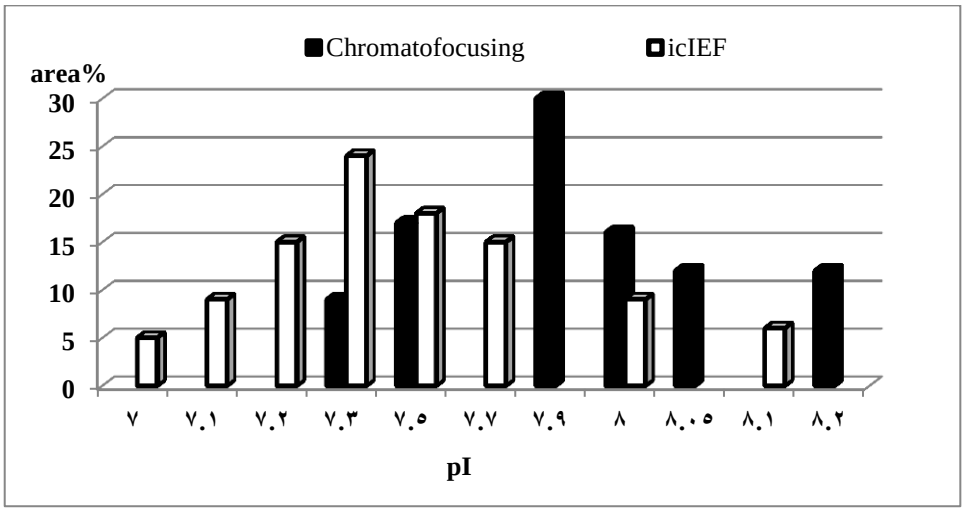


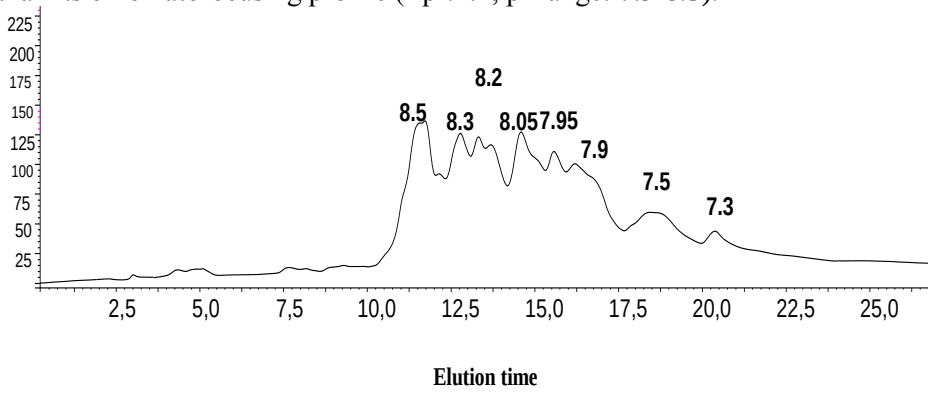
Figure 5: Comparison of % area of conjugated mAb-1 charge isoforms obtained by two methods (anion exchange chromatofocusing and icIEF)

Comparison of chromatofocusing and icIEF profiles of conjugated mAb-2

The mAb-2 was conjugated to a cytotoxic maytansine derivative through non-cleavable linker. The antitumor action of maytansinoid mAb-2 is based on the release of the maytansine derivative-linker which kills cancer cells by interfering with their division upon antibody/antigen binding and internalization. Ratio of maytansine derivative to mAb-2 is around *6.2* moles of maytansine derivative per mole of mAb-2.

Figure 6 demonstrated the chromatofocusing and the icIEF profiles of the conjugated mAb-2. As for the maytansinoid mAb-1, the conjugated mAb-2 was more heterogeneous than corresponding unconjugated antibody.

The icIEF profile of the conjugated mAb-2 was more heterogeneous (ΔpI:1.6, pI range: 7.4 – 9.0) than its chromatofocusing profile (ΔpI:1.1, pI range: 7.3-8.5).



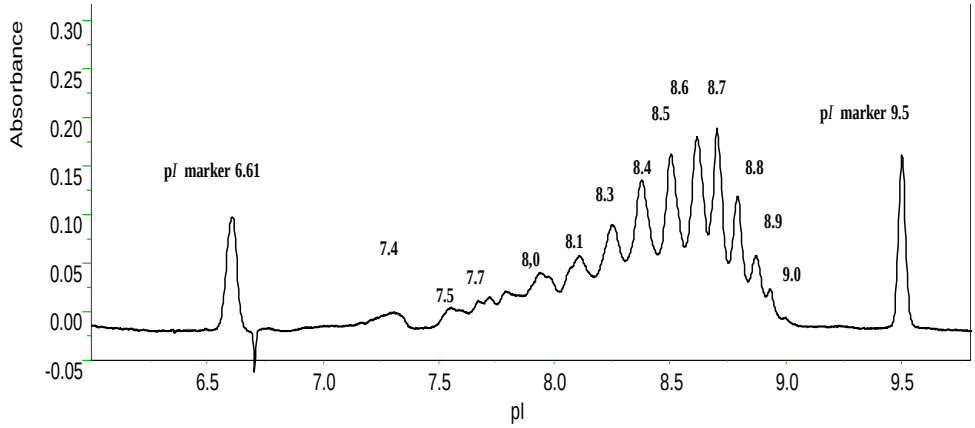


Figure 6: Chromatofocusing profile of conjugated mAb-2 (A): experimental conditions was as mentioned in material and methods, Injected quantity: 500µg. pI values: 8.5, 8.3, 8.2, 8.05, 8.0, 7.95, 7.9, 7.5 and 7.3.
icIEF profile of of conjugated mAb-2 (B). Final concentration of unconjugated antibody in sample matrix is 1mg/ml diluted in 0.35% methyl cellulose, 4% 3–10 pharmalytes/ 8– 10.5 pharmalytes (1:1 ratio), 2M urea. pI markers: 8. 18, 9.50. Focusing time :12min at 3000V.

The % area of charge variants obtained by the icIEF method were compared with those obtained by the chromatofocusing method (Figure 7). Charge variants of pI values 7.3, 8.05 and 8.2 were only observed by chromatofocusing, whereas charge variants of pI 7.4, 7.7, 8.6, 8.7, 8.8, 8.9 values and 9.0 were only observed by icIEF. With icIEF method, 4% and 1.7% of mAb-2 conjugate had pI values 8.9 and 9.0. These pI values revealed the presence of the unconjugated mAb in the ADC sample. icIEF profile showed the presence of unconjugated mAb (2%) while no charge variant correspond to the unconjugated mAb was presented in the chromatofocusing profile .

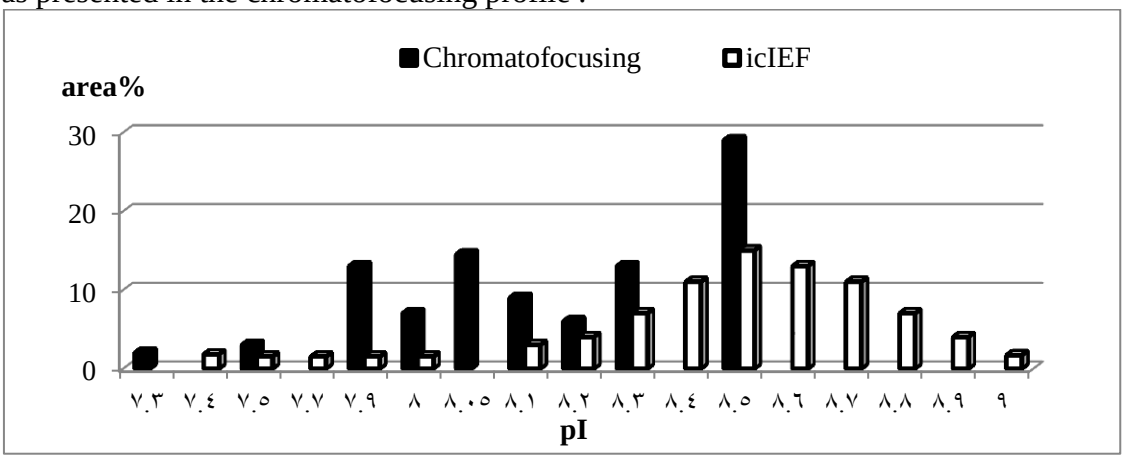


Figure 7: Comparison of % area of conjugated mAb-2 charge isoforms obtained by two methods (anion exchange chromatofocusing and icIEF)

Conclusion:

mAb and their drug conjugates (ADC) are a very important class of biopharmaceuticals. Monitoring charge variant profiles is one of the tools used to insure the quality of this therapeutic class. Chromatofocusing and icIEF are very suitable method for the separation and characterization of the charge variant profiles of unconjugated and conjugated antibodies. In this work, a comparison of charge variant profiles obtained by these two methods for two monoclonal antibodies (mAb-1 and mAb-2) and their drug conjugates was performed. The chromatofocusing charge variant profiles of studies antibodies and their conjugates (pI values and % areas of charge variants) were different from icIEF ones. As

expected, the separation of charge variants was achieved with good resolution using icIEF method compared to chromatofocusing method.

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