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ELECTROPHORESIS

RESEARCH ARTICLE

Dual-detection approach for a charge variant analysis of monoclonal antibody combination products using imaged capillary isoelectric focusing

Jason Candreva[1,#] | **Abbie L. Esterman**[1,#] | **Derek Ge**[2] | **Pritesh Patel**[1] | **Shannon C. Flagg**[1] | **Tapan K. Das**[1] | **Xue Li**[1]

[1]Biologics Development, Bristol Myers Squibb, New Brunswick, New Jersey, USA

[2]Pharmaceutical Sciences, University of Michigan, Ann Arbor, Michigan, USA

Correspondence
Xue Li, Biologics Development, Bristol Myers Squibb, 1 Squibb Drive, New Brunswick, NJ 08901, USA.
Email: xue.li1@bms.com

[#]The first two authors contributed equally to this publication.

Abstract

The clinical benefits of treatments with a combination of two or more therapeutic monoclonal antibodies (mAbs) have emerged in recent years. Imaged capillary isoelectric focusing is a frequently used technology in the biopharmaceutical industry for charge variant analysis of protein therapeutics. However, with the wide concentration ranges of combination products, one component may fall within the linear detection range, whereas the other does not. Here, we report a novel methodology to explore charge variants of mAb mixtures using multiple detection techniques simultaneously. We use ultraviolet absorbance to monitor the charge variants of the high-concentration component and native fluorescence (FL) to monitor the variants of the low-concentration one. Charge variants of mixtures that span 40-fold in ratio differences can be accurately quantified with this approach. In contrast to the conventional methods, it is not necessary to prepare and analyze two samples at different concentrations and combine the results for combination product testing. Additionally, the use of FL detection enables the charge variant analysis of highly potent/low abundant mAbs in a mixture. This methodology is more quality-control friendly and efficient for the charge variant analysis of combination products with wide ratios.

KEYWORDS

capillary electrophoresis, charge variants, combination therapy, iCIEF, monoclonal antibody

1 | INTRODUCTION

Therapeutic proteins manufactured in mammalian cell culture are a heterogeneous population, containing fragments, aggregates, and variants with post-translational modifications (PTMs), such as glycosylation, sialylation, deamidation, glycation, and oxidation [1–6]. These PTMs impact the charge of amino acid residues, resulting in changes of acid dissociation constant (p*K*a) and surface charge [6]. Control of charge variants has been an expectation from health authorities. Robust methods with high resolution are required to monitor the charge profile to support product and process development [2, 7, 8].

Compared to other technologies used to monitor charge variants, such as ion-exchange chromatography (IEX) [9–12], capillary zone electrophoresis (CZE) [12, 13], and traditional slab–gel isoelectric focusing (IEF) [14, 15],

Abbreviations: ABS, absorbance; FDC, fixed-dose combination; FL, fluorescence; ICH, International Conference on Harmonization; iCIEF, imaged capillary isoelectric focusing; QC, quality control; UV, ultraviolet.

imaged capillary IEF (iCIEF) offers a number of advantages for monitoring charge variants of biomolecules [7, 8, 12, 16–21]. The iCIEF technology is amenable to platform conditions for molecules with a wide range of p*I* values, as only minimal method development is needed for the transition amongst typical monoclonal antibody (mAb) molecules. Additionally, the introduction of dual detection of native fluorescence (FL) and ultraviolet (UV) provides a greater sensitivity of low abundant species with an increased dynamic linear range [12, 22, 23].

During iCIEF analysis, the separation occurs in a capillary and is monitored in real time using a charge-coupled device (CCD) camera [8, 22]. To mitigate protein aggregation and precipitation of biomolecules at their p*I*'s, additives such as urea are added to increase solubility [19, 21]. As the mobilization step is not required, the separation can be monitored in real time, decreasing the time for method development [17]. The data obtained from iCIEF analysis includes the apparent experimental p*I* of each isoform, as well as ratiometric quantification of each species. Typically, the percentages of acidic (lower p*I*) and basic (higher p*I*) variants as compared to the main isoform species are reported [6, 16, 18, 24, 25].

Recently, combination therapies have garnered increased efforts from the pharmaceutical industry enabling the development of patient-centric dosage forms, especially in the treatment of cancer. More than 1100 clinical trials of combination therapies have been documented for immuno-oncology treatments [26]. The treatment regimens include sequential [27–31], coadministration, or fixed-dose combination (FDC) products [32, 33]. For instance, immune checkpoint blockade of tumors is a novel strategy in clinical oncology using FDC products. In patients with advanced melanoma, treatment with relatlimab plus nivolumab FDC achieved a significant benefit compared to treatment with nivolumab monotherapy [32]. Additionally, the combination of pertuzumab and trastuzumab FDC to block HER2 gene overexpression in breast cancer has proven beneficial [33].

Aimed at both coadministration and FDC products, enhanced analytical methods are needed to monitor product quality attributes for use-time studies and program life cycles [34, 35]. The conventional iCIEF and IEX assays using UV detection lack the needed sensitivity [36] and dynamic range to simultaneously quantify charge variants of multiple components in wide ratios, such as FDC products with ratios ranging from 1:6 to 1:40. In a recent publication, Li et al. reported the use of FL detection to monitor charge variants of low levels of erythropoietin in simulated drug product formulations [37]. Another drawback of CIEF technology is lack of resolution among co-formulated molecules with similar p*I* values. Cao et al. reported a CIEF method for monitoring charge variants in co-formulated mAb products. However, peptide mapping was needed to quantify the acidic species of the higher p*I* mAb when the ratios were wider than 1:1 due to the closely related p*I* of the two mAbs, which could not be adequately resolved by CIEF [38]. Many of the challenges associated with traditional CIEF are also true of CZE, which makes the technology less than ideal in developing a method for robust analytical control. Goyon et al. reported a CZE method to quantify the charge variants in mAb mixtures, including a mixture of ipilimumab and nivolumab, but only at the 1:1 ratio [13].

In this paper, we discuss the development, qualification, and application of a novel iCIEF methodology to simultaneously and accurately quantify the acidic and basic charge variants of FDC products consisting of two mAbs with differing p*I*'s, with wide ratios up to 1:40. The limited dynamic range of sensitivity of conventional iCIEF methods is overcome by taking advantage of the dual-detection systems and the ability to simultaneously monitor native FL and UV absorbance. Due to the many inherent advantages of iCIEF technology and the dual-detection systems, the resulting method is QC (quality control)-friendly with suitable acceptance criteria for sensitivity, specificity, linearity, and repeatability, in accordance with the International Conference on Harmonization (ICH) guidelines [39].

2 | MATERIALS AND METHODS

2.1 | Materials

Maurice iCE systems with Compass software, methylcellulose, p*I* markers, Maurice electrolyte solution, and Fc cartridges were purchased from ProteinSimple (Santa Clara, CA). Urea was sourced from MilliporeSigma (St. Louis, MO) and Pharmalytes from GE Healthcare/MilliporeSigma (St. Louis, MO). Antibodies of IgG1 and IgG4 isotypes were provided by Bristol Myers Squibb. Empower v3 software was supplied by Waters (Milford, MA) and JMP software version 13.1.0. by SAS (Cary, North Carolina). Corresponding monotherapy antibodies were used as reference materials in this study for FDC mixtures.

2.2 | Methods

2.2.1 | iCIEF sample preparation

Individual mAb samples were diluted using ultrapure water (Milli-*Q*) to varying working concentrations (up to 10.0 mg/ml) for linearity experiments. For the analysis of FDC products, the individual mAbs were mixed at

differing mass ratios (from 40:1 to 1:40) and samples were prepared at a working concentration of up to 10.0 mg/ml. The samples were then diluted to 1:10 (final concentration up to 1.0 mg/ml) in molecule-specific master mixes consisting of 2–4 M urea, 0.35% methylcellulose, 4% Pharmalytes (mixtures of 3–10, 5–8, and 8–10.5), 5 mM arginine (only for mAb1:mAb3), and p*I* markers. The master mix component compositions were optimized using different ratios of Pharmalytes and additives for each pair of molecules to maximize resolution and ensure no overlap of the charge variants from the two molecules. Each new condition was further tested to verify that no changes in the relative abundance of the acidic, main, and basic regions were observed as compared to the values obtained using qualified methods for each molecule. For linearity studies, three independent preparations at each level were injected.

2.2.2 | Maurice instrument parameters

The separation occurs in a fluorocarbon-coated silica capillary (5-cm long with 100-μm inner diameter), and the entire capillary is monitored in real time using a CCD camera imaging with both native FL and/or absorbance at 280 nm. The Maurice instruments with Compass software were initialized as per vendor instructions. Samples were placed on the autosampler and maintained at 10°C during the run. The injections were prefocused at 1500 V for 1 min and focused at 3000 V for 10 min, respectively. Raw data was collected using dual-detection imaging of the whole capillary, simultaneously monitoring UV absorbance at 280 nm and native FL emission collected from 320 to 450 nm with 20-s exposure. Maurice separation and detection parameters were optimized during method development. Various exposure times were evaluated (5–20 s), with 20 s being optimal.

2.2.3 | Data analysis

Calibrated data from Compass software was exported to EMPOWER v3. Electropherograms, peak Integration, and peak area quantification of acidic variants, basic variants, and main peak area percentages were performed using EMPOWER v3. Linear regressions and fit analysis were performed using JMP.

3 | RESULTS AND DISCUSSION

With the emergence of coadministration and FDC biologic products, especially in the field of oncology, the need is critical for advanced analytical methods to monitor charge attributes in mixtures of multiple molecules. To our knowledge, this is the first report on a method to directly measure charge variants of combination products with ratios ranging from 1:1 to 1:40 using only iCIEF. The success of our methodology is demonstrated with proof-of-concept experiments using two model mixtures with different mAb combinations of IgG1 and IgG4 isotypes (Model 1: mAb1 and mAb2; Model 2: mAb1 and mAb3) mixed in ratios ranging from 1:40 to 40:1 (final concentrations range from ~0.02 to ~0.98 mg/ml). These model molecules represent mAbs with p*I* values ranging from 6 to 9.

3.1 | Limits of detection by UV absorbance

As shown in the electropherogram of the 1:1 mixture of mAb1:mAb2 (Figure 1), there is no overlap between the last basic peak of mAb2 and the first acidic peak of mAb1. It is important to ensure that no overlap of the variant peaks of mAb1 and mAb2 ensues, even in the case of peak drift or new peak formation due to a degradation event. Traditional UV detection is not sensitive enough for the lower concentration species in the FDC product with ratios wider than 1:6 to 6:1, whereas the signal of the more abundant species may have saturated the detector. However, as shown in Figure 1, the less-abundant mAb can be reliably quantified using FL detection. By taking advantage of the dual detection, the charge variants of each mAb in the wide ratio FDC product can be quantified in a single injection. Additionally, the use of FL detection eliminated the known interference of histidine in formulation buffer using UV detection, as histidine has negligible signal at the 320–450 nm wavelength range used in native fluorescent mode [40, 41].

3.2 | Dynamic linear range of absorbance and native fluorescence

Using the traditional iCIEF approach, in order to analyze ratios wider than 1:6 and 6:1, two sample preparations are required — one to quantify the more abundant species at the nominal sample concentration; and a second at a higher sample concentration in order to have enough signal for the less abundant species, meanwhile saturating the detector of the more abundant species. This traditional protocol has two disadvantages: (1) Significantly overloading the capillary with a high-concentration sample often results in sample precipitation and capillary clogging; and (2) it is not QC-friendly as the analyst has to prepare and analyze two samples and combine the data for meaningful

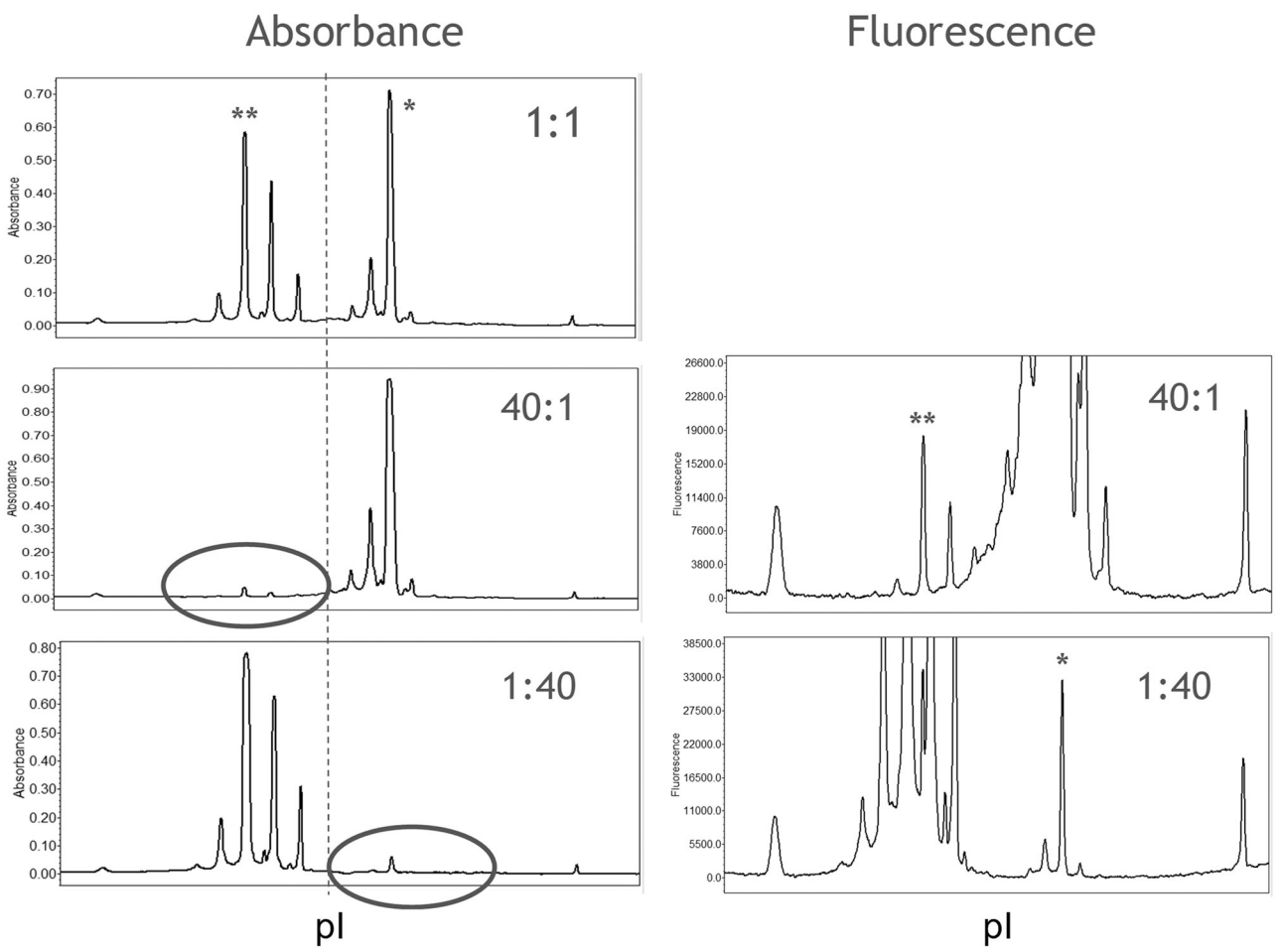


FIGURE 1 Analysis of mAb1:mAb2 mixtures with imaged capillary isoelectric focusing (iCIEF) using dual detection of absorbance at 280 nm and fluorescence. The areas in the circle are the expected regions of the charge variant peaks of the lower abundant species in the 40:1 and 1:40 mixtures, which are not quantifiable using absorbance. These species can be quantified using fluorescence. Asterisk (*) and double asterisk (**) represent the locations of the main peak of mAb1 and mAb2, respectively.

results. Both concerns can be alleviated by employing our proposed dual detector approach.

The advantage of monitoring the capillary with absorbance and FL simultaneously is portrayed in Figure 1. However, preliminary evaluation is needed to determine which detection mode to use for the quantification of each component in the mixture. In some cases, where the ratio is closer to 1:1, absorbance will be sufficient to quantify both components. With wider ratio products, between 1:6 and 1:40, FL detection may be required to accurately quantify the lower abundant species. In order to determine which detection mode is appropriate, linearity is performed with both absorbance and FL. The concentration of the component to be measured should be within the acceptable linear range of the detection mode, taking into consideration the lower abundance of acidic and basic variants, to ensure method robustness within the expected performance variance. In this study, the upper limit for absorbance linearity of mAb1 and mAb2 is ~1.0 mg/ml, and 0.667 mg/ml for mAb3 due to its higher extinction coefficient.

As an example, Figure 2 shows the representative electropherograms of mAb3 across the entire linear range, which ranges from 0.088 to 0.667 mg/ml for UV, and 0.017 to 0.088 mg/ml for FL. The end points of the range (0.017–0.667 mg/ml) represent 1:40 dilution. The electropherograms show that the profiles and relative percent peak areas are consistent across the mass range using both detection modes. Data qualifying the method following ICH guidelines for repeatability, accuracy, and precision are presented later.

Experiments were performed to determine the linear range of each component of the mAb mixtures. The criteria assessed include visually comparable profiles, the accuracy of 80%–120% recovery (the recovery is calculated by comparing experimental values to theoretical values determined by the regression line), precision (RSD $\leq$ 5.0% for main peak area; RSD $\leq$ 15% for peak area of variant

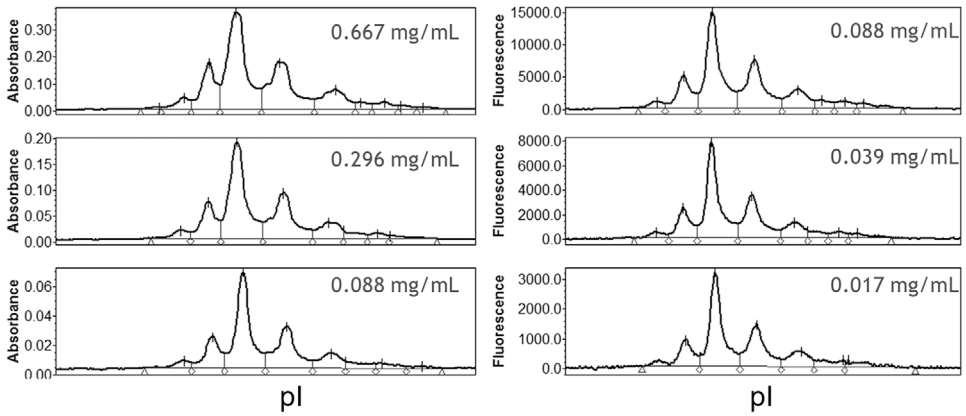


FIGURE 2 Electropherograms of imaged capillary isoelectric focusing (iCIEF) analysis of mAb3 covering sample concentrations of 0.017–0.667 mg/ml. The upper portion of the range (left, 0.088–0.667 mg/ml) was acquired with absorbance detection at 280 nm. The lower portion of the range (right, 0.017–0.088 mg/ml) was acquired with native fluorescence detection.

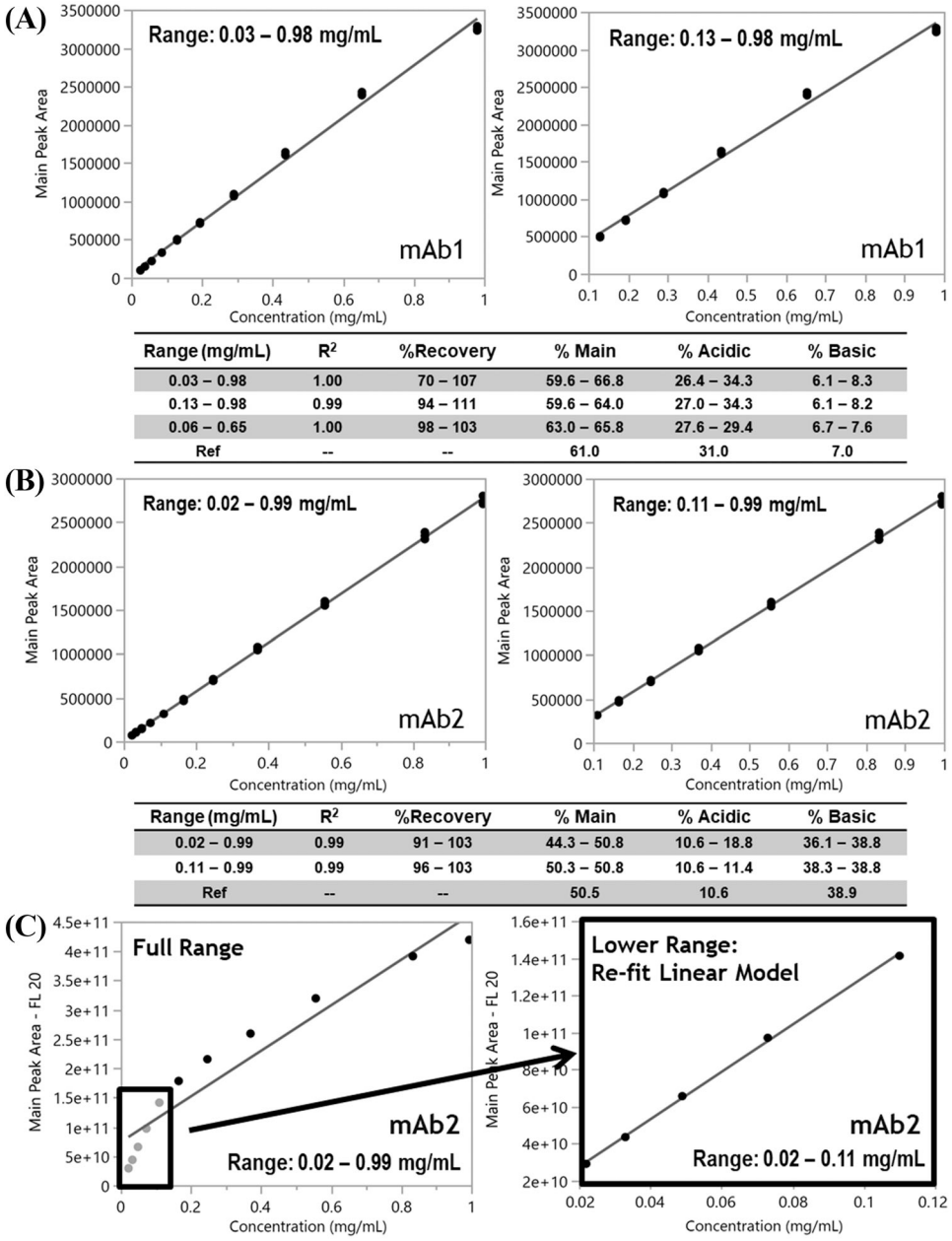
species), and linearity ($R^2 \geq 0.98$). The absorbance linearity of the main peak area of mAb1 and mAb2 is shown in Figure 3A,B. Although the data appears to be linear across wide concentration ranges ($R^2 \geq 0.99$), there is a loss of accuracy at lower and upper concentrations of the ranges. By narrowing the ranges at the lower concentrations (e.g., 0.13 mg/ml instead of 0.03 mg/ml for mAb1; 0.11 mg/ml instead of 0.02 mg/ml for mAb2), recovery is improved. Furthermore, by narrowing the concentration range of mAb1 from 0.03–0.98 to 0.06–0.65 mg/ml, the recovery of the main peak improved from 70%–107% to 98%–103%. This is because the signal is now within the linear range of the UV detector and is an example of how R^2 is not the only indicator of linearity across large mass ranges. Similarly, for mAb2, the percent recovery was improved by narrowing the concentration range.

The FL linearity was evaluated using 20 s of exposure across 0.02–0.99 mg/ml concentration range. FL linearity of the mAb2 main peak is shown in Figure 3C. Across the entire range, the signal begins to get saturated at high concentrations. However, the signal is linear at the low concentrations of the range 0.02–0.11 mg/ml, as shown by the zoomed-in view, and the lower concentration range was determined to be linear as shown in Table 1. The linear range for each detection mode, determined from both the absorbance and FL data, is also shown in Table 1. These results demonstrate the feasibility of using absorbance for charge variant analysis at relatively higher concentration ranges and FL at relatively lower concentration levels.

3.3 | Proof-of-concept 1:40 to 40:1 FDC

Once the linearity ranges with both detection modes have been determined, the evaluation of the method performance parameters is the next key step. To demonstrate the applicability of this method to accurately quantitate wide ranges of mAb mixtures, two mAb combinations ranging from 1:40 to 40:1 were tested. The results of the mixtures of mAb1 and mAb2 are shown in Figure 4A. FL detection was used in cases where one species was significantly less abundant than the other, as denoted by the asterisk in Figure 4A. The FL values fall within the linear range, which was determined for each molecule during the method development phase. For mAb1 and mAb2, the ranges were selected based on the previous studies on the linearity of each detection mode. Across the entire range, the quantification of the acidic, main, and basic peaks of each component was accurate and repeatable. Three independent preparations at each level resulted in RSD values ≤4.9% for all variant species measured and ≤0.7% for the main peak. The RSD values are well within the typical repeatability acceptance criteria of 15.0% and 5.0%, respectively. Accuracy also passed typical acceptance criteria (% recovery of 80–120 for the main peak and 70–130 for variant species). The acceptance criteria are based on an internal guideline for method qualification.

The results for FDC products of mAb1 and mAb3 are shown in Figure 4B. The RSD values were ≤4.4% for all variant species measured and ≤1.6% for the main peaks (see the previous paragraph for the acceptance criteria). For this pair of mAbs, the acceptable range is limited to FDC ratios of 1:25 to 40:1. The absorbance linearity range of mAb3 did not support concentrations of mAb3 in FDC products wider than 1:25 (i.e., 1:40), as mAb3 saturates the UV detector at concentrations above 0.667 mg/ml. To accurately measure ratios below 1:25, a dilution of the sample would be needed with additional method optimization. This is a good example demonstrating the necessity for independent linearity evaluation of each molecule to adapt to different mAb mixtures in FDC products with wide ratios.



Range (mg/mL)	R^2	%Recovery	% Main	% Acidic	% Basic
0.03 – 0.98	1.00	70 – 107	59.6 – 66.8	26.4 – 34.3	6.1 – 8.3
0.13 – 0.98	0.99	94 – 111	59.6 – 64.0	27.0 – 34.3	6.1 – 8.2
0.06 – 0.65	1.00	98 – 103	63.0 – 65.8	27.6 – 29.4	6.7 – 7.6
Ref	--	--	61.0	31.0	7.0

Range (mg/mL)	R^2	%Recovery	% Main	% Acidic	% Basic
0.02 – 0.99	0.99	91 – 103	44.3 – 50.8	10.6 – 18.8	36.1 – 38.8
0.11 – 0.99	0.99	96 – 103	50.3 – 50.8	10.6 – 11.4	38.3 – 38.8
Ref	--	--	50.5	10.6	38.9

FIGURE 3 Absorbance linearity of main peak area for (A) mAb1 and (B) mAb2. Both mAbs show good linearity R^2 values across the entire final concentration range. However, by narrowing the range, recovery is improved for both mAbs. The values of the main, acidic, and basic relative area percentages are closer to the reference material (Ref) with the improved recovery compared to the wide range of linear fits. Ranges of percent peak areas for acidic, main, and basic are given for data obtained across the concentration range. (C) Fluorescence linearity with 20-s exposure for the main peak area of mAb2. From 0.02 to 0.99 mg/ml (final concentration), the signal gets saturated at high concentrations. The data is linear at the lower concentration range from 0.02 to 0.11 mg/ml, as shown in the refit model.

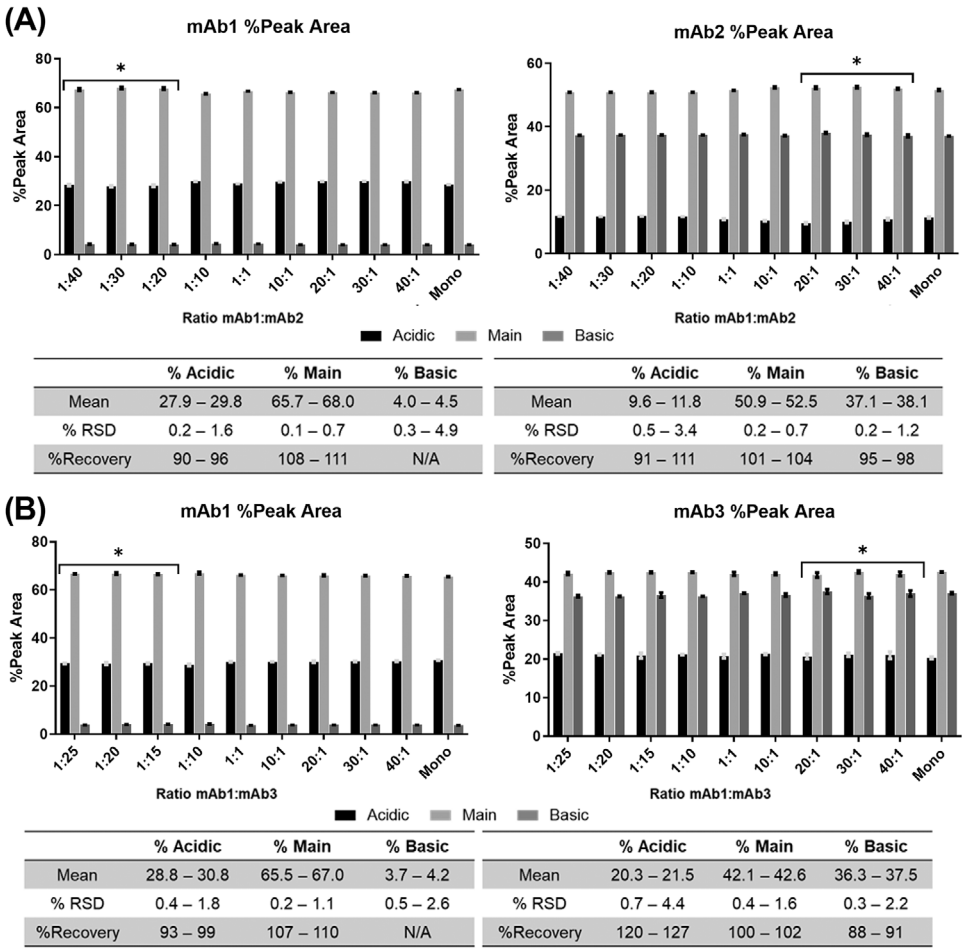
For the novel methodology reported in this paper, the two molecules in combination did not show any overlapping peaks due to their dissimilarities in p*I*, thus, facilitating the data analysis of the charge variants belonging to each molecule. We are currently evaluating strategies to analyze FDC products containing molecules with p*I*'s close in value. In one approach, where overlapping charge variant peaks cannot be resolved, peak grouping can be utilized to track changes in charge variants for QC testing. The drawback to this approach is the inability to distinguish molecule-specific changes in the mixture. With the emergence of new technologies, such as Blaze and CEInfinite with direct iCIEF to mass spectrometry sample injection, peak identification and characterization may be possible [42–44]. A second approach is to optimize the master mix components, including the use of chemical "spacers,"

TABLE 1 Linear concentration ranges for mAb1, mAb2, and mAb3 with absorbance (ABS) and fluorescence (FL) with 20 s exposure

	Range (mg/ml)	Main peak R^2	% Recovery	% Acidic	% Main	% Basic
mAb1 ABS	0.13–0.98	0.9937	94–111	27.4–33.9	59.1–66.8	7.1–8.2
mAb1 FL[a]	0.03–0.13	0.9999	98–102	28.7–31.1	61.1–63.8	7.4–7.8
mAb1 Ref	–	–	–	31.0	61.0	7.0
mAb2 ABS	0.11–0.99	0.9989	96–103	10.6–11.4	50.3–50.8	38.3–38.8
mAb2 FL[a]	0.02–0.11	0.9991	97–102	10.3–11.0	49.2–50.6	39.1–39.8
mAb2 Ref	–	–	–	10.6	50.5	38.9
mAb3 ABS	0.06–0.67	0.9983	91–105	16.9–18.5	41.5–42.6	39.2–41.2
mAb3 FL[a]	0.02–0.06	0.9978	99–102	15.4–16.7	41.8–42.7	41.4–42.6
mAb3 Ref	–	–	–	16.9	41.9	41.2

Note: Ranges of relative percent peak areas for acidic, main, and basic are given for data obtained across the final sample concentration range (after master mix addition). Ref values reported from the Certificate of Analysis.

[a]Fluorescence.



	% Acidic	% Main	% Basic
Mean	27.9 – 29.8	65.7 – 68.0	4.0 – 4.5
% RSD	0.2 – 1.6	0.1 – 0.7	0.3 – 4.9
%Recovery	90 – 96	108 – 111	N/A

	% Acidic	% Main	% Basic
Mean	9.6 – 11.8	50.9 – 52.5	37.1 – 38.1
% RSD	0.5 – 3.4	0.2 – 0.7	0.2 – 1.2
%Recovery	91 – 111	101 – 104	95 – 98

	% Acidic	% Main	% Basic
Mean	28.8 – 30.8	65.5 – 67.0	3.7 – 4.2
% RSD	0.4 – 1.8	0.2 – 1.1	0.5 – 2.6
%Recovery	93 – 99	107 – 110	N/A

	% Acidic	% Main	% Basic
Mean	20.3 – 21.5	42.1 – 42.6	36.3 – 37.5
% RSD	0.7 – 4.4	0.4 – 1.6	0.3 – 2.2
%Recovery	120 – 127	100 – 102	88 – 91

FIGURE 4 Proof-of-concept imaged capillary isoelectric focusing (iCIEF) method for fixed-dose combination (FDC) products of (A) mAb1:mAb2 from 1:40 to 40:1 and (B) mAb1:mAb3 from 1:25 to 40:1. In (A), data is shown for mAb1 (left) and mAb2 (right) percent peak area. In (B), data is shown for mAb1 (left) and mAb3 (right) percent peak areas. %RSD values (presented as error bars) are calculated from three independent preparations of each ratio. Minimum and maximum ranges are given for mean and %RSD values from results obtained across all ratios. Due to the low area percent of the basic group of mAb1, the percent recovery is reported as "N/A." Asterisk (*) designates data obtained with fluorescence with 20-s exposure; all other data obtained with absorbance at 280 nm.

to further separate the two molecules for analysis. However, this may be challenging due to lack of appropriate reagents to provide the necessary resolving power.

4 | CONCLUDING REMARKS

Leveraging simultaneous monitoring of FL and UV absorbance, the iCIEF methodology proposed herein enables a concurrent quantification of charge variants for FDC products with differing p*I*'s and wide ratios up to 1:40. The qualification of the novel method included repeatability, linearity, and accuracy (recovery) modules. Determining linearity for both detection modes is required for each component in the FDC products during method development. This methodology avoids sample precipitation or capillary clogging issues caused by sample overloading using conventional protocols with only UV detection. This approach requires only a single sample preparation; therefore, the analyst does not need to prepare and analyze two different sample dilutions and then combine the results. This provides an efficient, reliable, reproducible, and QC-friendly method for combination product testing.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Xue Li https://orcid.org/0000-0002-9850-5132

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