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Charge Variant Analysis of Therapeutic Monoclonal Antibodies Using a pH Gradient Generated by Auto•Blend Plus

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APPLICATION BENEFITS

- Increased productivity through the automation of analytical method parameter evaluations
- Thorough method development for charge separation using pH gradients for confirmation and quantification of biotherapeutic charge variants
- Efficient pH or ionic strength screening using concentrated stocks

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP SCX Column

KEY WORDS

Auto•Blend Plus,™ cation exchange, antibody, IEX, SCX, bioseparation, therapeutic protein, method development, pH gradient, UPLC

INTRODUCTION

Charge-based separation methods play an important role in characterization studies and quality control strategies for biotherapeutics.[1-4] For the analysis of charged species of antibodies, ion exchange chromatography (IEX) has a widespread use in the biopharmaceutical industry for its ability to resolve species related to protein conformation, size, sequence variants, glycosylation, and post-translational modifications. The capability to perform protein characterization under non-denaturing conditions combined with the ability to isolate charge variants easily has both contributed to the popularity of IEX in charge variant analysis of biotherapeutics.

Protein separations by IEX methods routinely utilize salt (ionic strength) or pH gradients to elute the protein from the IEX column. Although somewhat different in the separation mechanisms of the two eluting methods, method parameters such as column types, mobile phase composition, and pH (or salt concentration) gradients often need to be evaluated to yield the optimal separation for each individual antibody.[5] However, the evaluation of the selected method parameters often requires a time consuming, iterative process that involves preparing and testing discrete buffers of varying compositions. This requirement imposes a great challenge to the method development process, and calls for an intelligent setup/process that decreases time spent on method development, and improves the efficiency of the workflow.

Auto•Blend Plus Technology uses the ACQUITY UPLC H-Class System's quaternary solvent manager to blend individual pure solutions and concentrated stocks from the reservoirs to deliver pH gradients for the separations of charge variants in therapeutic proteins. The technology allows the analyst to evaluate multiple buffer compositions from the concentrated stocks; enabling the evaluation process to be easily automated to increase the productivity of charge variant analysis. Auto•Blend Plus Technology allows for the use of mixed buffers with different pka values to increase the buffering capacity and extend the effective working pH range that a single buffering species cannot deliver.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class System with Auto•Blend Plus
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm (p/n 186004930)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	3 µL
Flow rate:	0.50 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM sodium phosphate dibasic
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Buffer concentration:	20 mM
Gradient:	pH 5.2 to 7.9 in 30 minutes (Figure 2); pH 6.5 to 7.2 in 15 minutes (Figure 6)

Informatics for data collection and processing

UNIFI Scientific Information System, v 1.6

In addition to these benefits, the unique design of Auto•Blend Plus Technology provides the flexibility to allow analysts to switch between pH or salt gradients during the method development process for the determination of optimal separation parameters. The objective of this application note is to demonstrate the performance of Auto•Blend Plus for optimizing IEX methods for charge variant separations using pH gradients. A therapeutic monoclonal antibody, infliximab, was used as a model protein to evaluate the functionality.

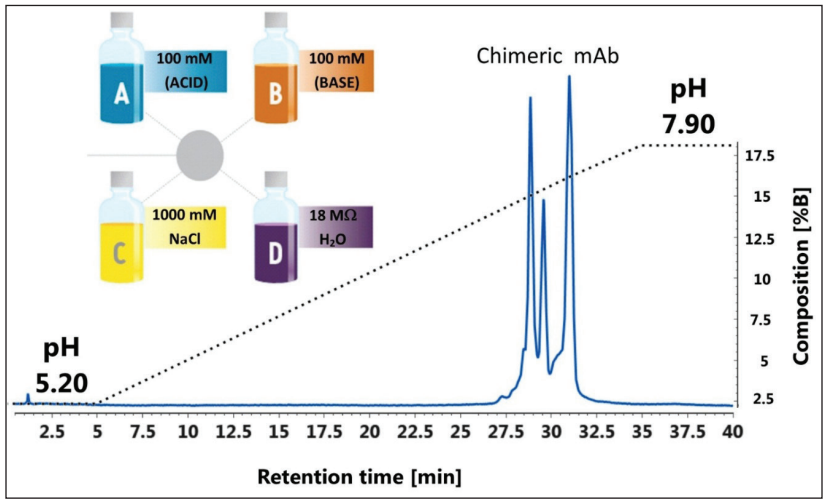

Figure 1. Automated delivery of an extended pH gradient range by the Auto•Blend Plus Technology. The chromatogram shows a gradient spanning from pH 5.20 to 7.90 is formed for the separation of lysine truncation charge variants of a chimeric monoclonal antibody (infliximab).

Sample Preparation

A Waters Protein-Pak Hi Res SP, strong cation exchange column (7 µm, 4.6 x 100 mm, p/n 186004930) was conditioned as outlined by the manufacturer. MES monohydrate (p/n A69892), sodium phosphate dibasic (p/n S5136), and sodium chloride (p/n S1679) were purchased from Sigma Aldrich. The pH gradients generated by Auto•Blend Plus were monitored on-line using a GE Healthcare Monitor pH/C-900 similar to previous work (p/n 720004149en). Calibration was performed at flow rates of 1 mL/min with the column off-line using the reference pH values from the empirical table data. The mAb samples evaluated in this study were used as received for all experiments at a concentration of 20 µg/µL.

RESULTS AND DISCUSSION

Flexible method development with the Auto•Blend Plus

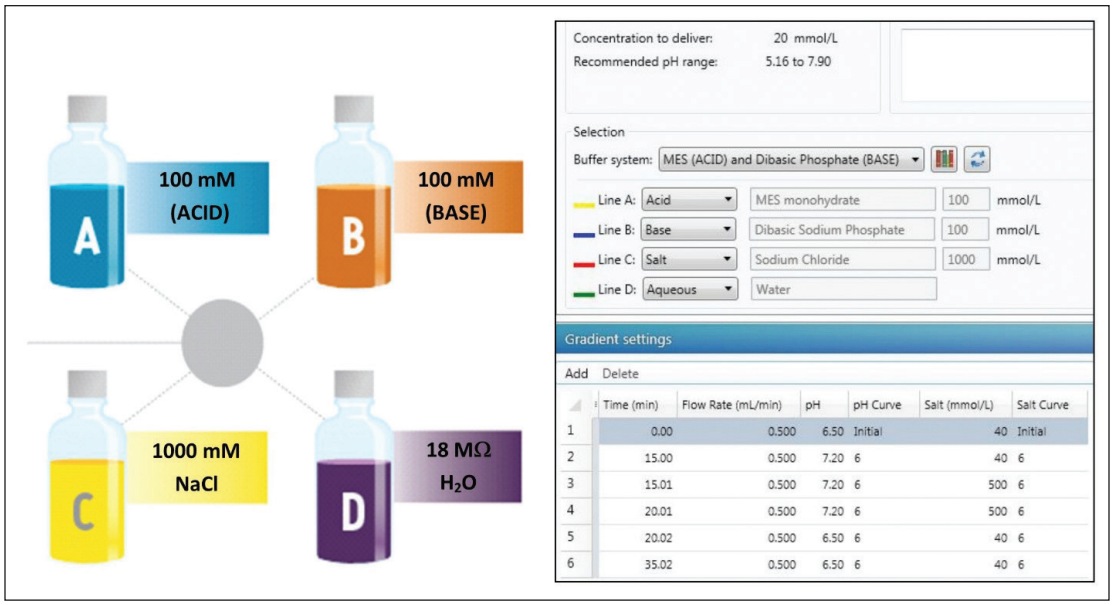

Figure 2. An example of the intuitive interface for programming pH or salt gradients rendered by the Auto•Blend Plus Technology. The software algorithm takes in the specified values and automatically calculates the percentage of acid and base required to deliver the desired pH gradient range and ionic strength.

Figure 2 shows a pH gradient table that Auto•Blend Plus Technology is programmed to generate using pure solutions and concentrated stocks on a quaternary solvent management system. The gradient table presents an easy-to-use interface to the end user, where the gradient is expressed directly in terms of pH and ionic strength. The software algorithm can independently control pH or ionic strength enabling the analyst to generate a variety of gradient conditions including constant pH with varying ionic strength, vice versa, or change pH and ionic strength simultaneously. With Auto•Blend Plus, the software automatically calculates the percentage of acid and base required for each pump stroke to deliver the specified pH using the chosen buffer system. Auto•Blend Plus allows for multiple buffer compositions to be mixed and evaluated from a single set of pure components, reducing cost and time in method development.

Increasing productivity with custom buffer systems

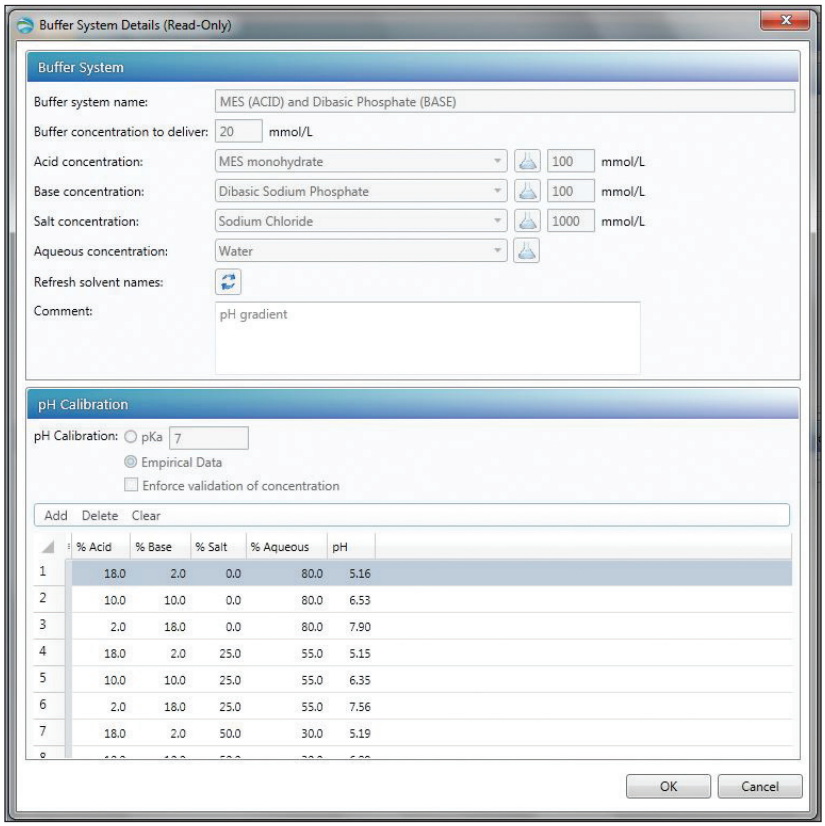

Figure 3. An example of the empirical table used by Auto•Blend Plus. Nine buffer mixture standards were used to create the exemplary reference table from which Auto•Blend Plus is based to automatically deliver a pH range over 5.2 to 7.9.

Traditional ion exchange chromatography employs buffers comprised of the same molecular species such as MES buffer (pH 5.5 to 6.7), phosphate buffer (pH 6.7 to 7.6), and HEPES buffer (pH 7.6 to 8.2). The limited working pH ranges of these individual buffer systems prolong the method development process for a separation based on pH gradients since multiple buffers need to be prepared and tested to optimize the separation performance over the entire pH range.

Auto•Blend Plus Technology allows for the preparation of custom buffers with an extended working pH range through the use of the empirical calibration table as shown in Figure 3. For this work, a 100 mM solution of 2-ethanesulfonic acid monohydrate (MES monohydrate) was prepared as the acidic reservoir and a 100 mM solution of sodium phosphate dibasic was prepared as the basic reservoir as illustrated in Figure 2. The empirical table was constructed from nine buffer mixture standards prepared from the concentrated stocks. The pH value was measured with a pH meter and entered into the table as shown in Figure 3. The use of MES monohydrate and sodium phosphate dibasic as an ion exchange buffer system gives an extended working pH range of 5.2 to 7.9, allowing for a larger set of experimental parameters to be tested from a single set of buffers.

The ability to generate extended pH ranges from concentrated stock buffers via Auto•Blend Plus without the need for multiple buffer systems makes it ideal for increasing productivity and reducing method development costs.

Linear pH gradients with Auto•Blend Plus

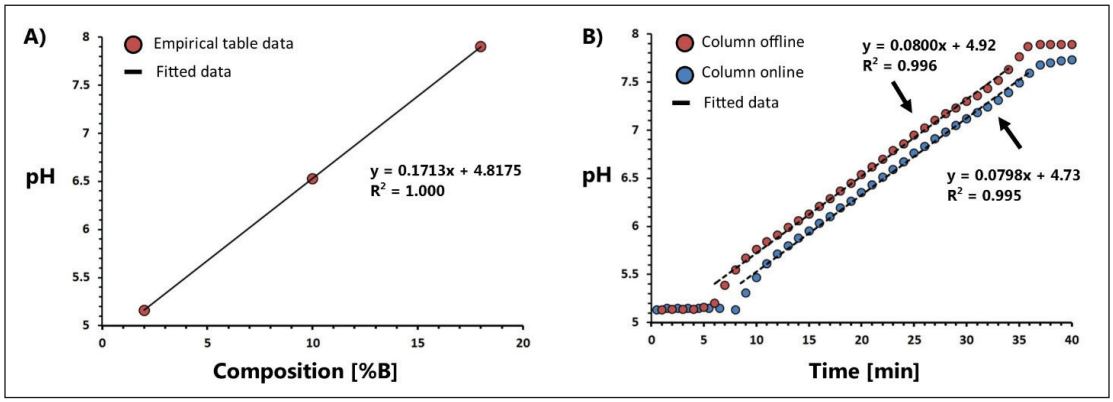

Figure 4. Using the A) empirical table data, Auto•Blend Plus was programmed to generate a 30 minute gradient from 5 to 35 minutes with a pH value ranging from 5.20 to 7.9. B) The generated gradient was evaluated for pH linearity in the presence or absence of the IEX column in the LC system. Each point in the figure was acquired using an on-line pH meter for the buffer composition (gradient) generated by Auto•Blend Plus.

The fidelity of a pH gradient generated by Auto•Blend Plus was assessed for its ability to produce a designed linear pH gradient over an extended pH range using a selection of buffer systems. For illustrative purposes, a plot of pH versus composition %B (% Base) was constructed from the empirical table data shown in Figure 3 to assess pH response linearity for the selected buffer system. From Figure 4A it can be seen that the empirical data for the chosen buffer system has a linear response over a pH range of 5.2 to 7.9. Auto•Blend Plus was programmed to generate a 30-minute gradient over a pH range of 5.2 to 7.9 starting at the 5-minute mark using the empirical table data from Figure 3. The programmed gradient was evaluated with or without the Waters Protein-Pak Hi Res SP, a strong cation exchange column, online so the impact of column effects on the variation of pH linearity could also be assessed.

Similar to a method from previous work[6] the mobile phase pH was monitored in-line using a GE Healthcare monitor pH/C-900 with pH being manually recorded in 1 minute intervals. From Figure 4B it can be seen that Auto•Blend Plus is capable of delivering a highly linear pH gradient over the gradient time window using the extended pH buffer system. The close agreement of the slopes of the fitted data on both plots indicates the column had no significant effect on the linearity of the pH gradient other than a small time delay due to the additional volume introduced by the column. The ability to deliver an extended linear pH gradient using custom buffer selections makes Auto•Blend Plus well suited for flexible method development.

Automation for pre-screening experimental method parameters

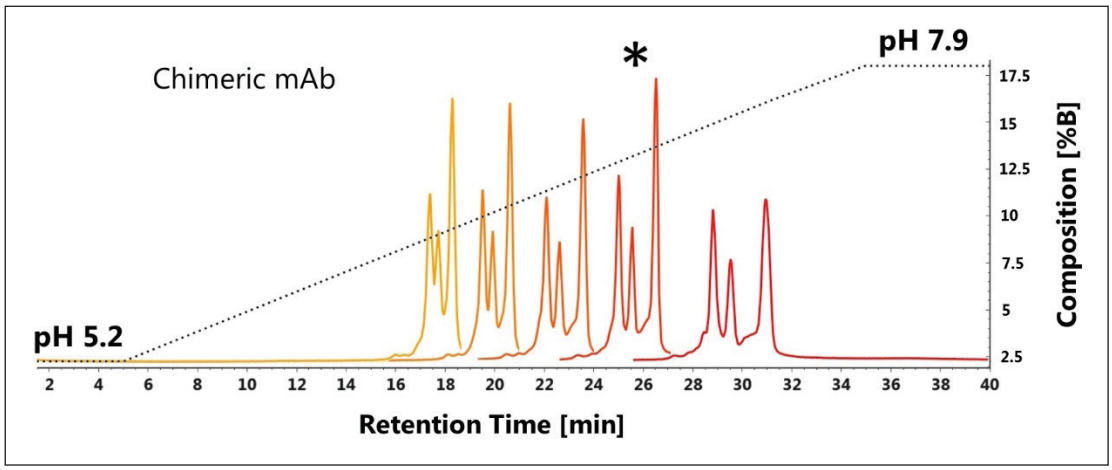

Figure 5. Optimization of ionic strength in the separation of lysine charge variants of a chimeric monoclonal antibody. From right to left, the ionic strength tested for each chromatogram was 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM NaCl. The ionic strength of 40 mM (denoted by the asterisks) provides the highest resolution between the main lysine variant peaks.

Method development of IEX separations often involves a time-consuming trial and error procedure because a successful separation cannot be readily predicted. The iterative process involves preparing multiple buffers at a specific pH and ionic strength, followed by testing each buffer system for separation performance until an adequate separation is achieved. The ability of Auto•Blend Plus Technology to blend multiple buffer compositions from a single set of concentrated stocks allows for evaluation of many experimental parameters in a highly efficient manner.

For example, it is well known that ionic strength affects separation performance, and that it should be evaluated in the optimization process. Figure 5 shows how Auto•Blend Plus Technology was used to evaluate the impact of ionic strength on a mAb charge separation with the extended pH buffer range prepared earlier. From the concentrated stocks the ionic strength (line C) was increased in 20 mM intervals for each chromatographic trace shown in Figure 5, starting with 20 mM on the right side of the plots.

Using the chromatographic peak that represents the charge variant of the monoclonal antibody containing two C-terminal Lysine residues as the investigative target (see Figure 6), we systematically evaluated the impact of ionic strength on the charge separation performance during our experiments. Resolution for the +2 Lys peak was reported as 2.66, 2.70, 2.33, 1.77, and 1.28 corresponding to the ionic strengths of 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM, respectively. This automated evaluation process renders an efficient and consistent way to find the optimized ionic strength for the pH gradient slope, and demonstrates that Auto•Blend Plus is an integrated software solution designed to streamline method development.

Integration with advanced informatics for automated data acquisition, processing, and reporting

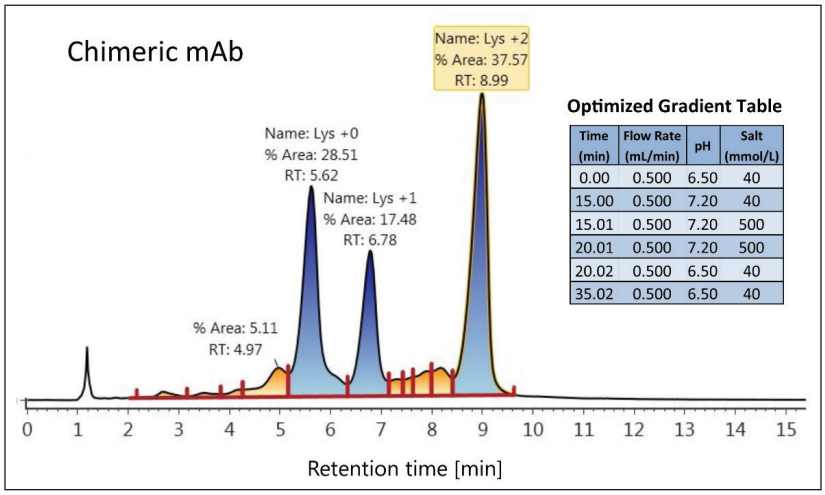

Figure 6. An optimized high throughput pH gradient separation of a chimeric monoclonal antibody in under 10 minutes. The pH gradient was optimized from pH 6.50 to 7.20 with ionic strength constant at 40 mM NaCl. Identification and integration of the C-terminal lysine variants were performed using tools within UNIFI Software and automatically displayed in the chromatogram window.

The Waters Biopharmaceutical Platform Solution with UNIFI provides a highly efficient analytical platform solution for routine characterization of biotherapeutics. Auto•Blend Plus Technology combines with other informatics tools within UNIFI Software to deliver an integrated workflow to perform charge-based separation and optimization using pH gradients. This workflow possesses the capability to automatically acquire data, process the data and provide a report, further enhancing and streamlining the method development process. Utilizing the informatics tools from UNIFI, further optimization of the pH gradient was performed.

Examination of the separation results from Figure 5 suggests that the pH gradient range that would effectively deliver the same separation performance observed at 40 mM NaCl would only require a portion of the 40-minute run time. Since the pH gradient curve generated by Auto•Blend Plus Technology follows a predictable mathematical equation, the pH value at any point along the gradient can be readily calculated and changed so equivalent separation performance can be achieved with shorter analysis time.

Using this methodology, the separation achieved in Figure 5 at an ionic strength of 40 mM was further optimized with the elution of the full charge variant profile of the chimeric monoclonal antibody within 10 minutes as shown in Figure 6. Relative peak area, retention time, and component name of the chromatographic peaks of interest were automatically calculated and labeled by the UNIFI Software. Quick delivery of the information by UNIFI to evaluate the separation performance is time-saving and promotes the method development process.

CONCLUSIONS

The development of methods for the analysis of biopharmaceutical charge heterogeneity profiles is a time-consuming process that requires methods that can be automated, quickly adapted, and readily deployed to meet the demands of the biopharmaceutical industry. The combination of Auto•Blend Plus Technology with the ACQUITY UPLC H-Class System improves workflow efficiency by allowing multiple buffer compositions to be tested from a single set of pure components. The flexibility to work with pH or salt gradients, combined with the ability to automate, makes Auto•Blend Plus Technology a powerful tool for increasing productivity and reducing development costs.

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