A design of experiment (DoE) approach to facilitate capillary isoelectric focusing method development for Hemlibra

Featuring the BioPhase 8800 system

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This technical note demonstrates a multifactorial DoE approach to facilitate high-speed capillary isoelectric focusing (cIEF) method optimization for the bispecific humanized monoclonal antibody, Hemlibra. The combination of multi-capillary CE equipment from SCIEX and a multifactorial DoE tool enabled the evaluation of 4 factors and 26 samples in only 6 hours. This approach significantly reduced the method development time from weeks to 1 day.

The traditional one-factor-at-a-time (OFAT) method development approach is tedious and time-consuming. The OFAT approach is further complicated when it is applied to a separation mechanism, such as cIEF, that is dependent on many different parameters. Multifactorial DoE enables the streamlined evaluation of multiple factors in a single set of experiments. The BioPhase 8800 software can then be used to statistically compare all factors used in the dataset and overcome the laborious process of OFAT.

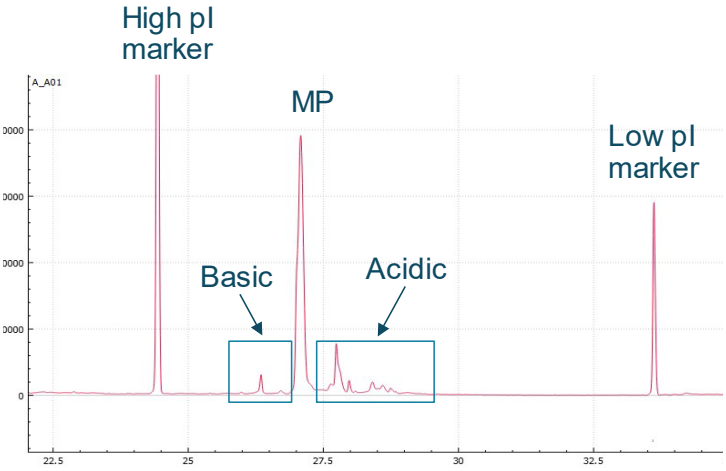

Figure 1. cIEF profile of Hemlibra after DoE methods optimization.

In cIEF experiments, multiple separation and sample preparation variables complicate method optimization. For example, the sample concentration, amount of anodic and cathodic stabilizers, urea concentration and ampholyte composition can affect cIEF separation resolution and reproducibility. In addition, separation parameters, including the duration and the voltage used during the focusing and mobilization steps, can impact the cIEF profile.

This technical note describes a strategy to minimize method development, optimization efforts and time investment by combining a multifactorial DoE tool and a multi-capillary analytical platform.

Key features of the DoE approach on the BioPhase 8800 system

- **Fast method development:** A combination of multi-capillary CE and multifactorial DoE facilitates method development in 1 working day
- **High reproducibility:** The BioPhase 8800 system provides excellent reproducibility (%RSD) for pI and peak area percentage composition at or below 0.02% and 3.4%, respectively
- **Improved resolution:** A resolution of 2-5 was achieved between the basic variant and the main peak and a resolution of 2-4 was achieved between the acidic variant and the main peak (Figure 1)

Methods

Materials: The BioPhase Capillary Isoelectric Focusing (cIEF) kit (P/N: C30101), containing the cIEF cathodic stabilizer, cIEF anodic stabilizer, cIEF gel, cIEF urea, cIEF anolyte, cIEF neutral capillary conditioning solution, cIEF catholyte, cIEF chemical mobilizer, cIEF formamide and CE-grade water, was from SCIEX (Framingham, MA). The BioPhase sample plates (P/N: 5080313), BioPhase reagent plates (P/N: 5080314), BioPhase outlet plates (P/N: 5080315), BioPhase neutral capillary cartridge (8 cm x 30 cm, 50 µm inner diameter, P/N: 5080119) and cIEF peptide marker kit (P/N: A58481) were from SCIEX (Framingham, MA). The 30 mg/mL, single-dose vial of Hemlibra was purchased from Myonex (Horsham, PA). The Pharmalyte narrow range (pH 5–8) ampholytes (P/N: 17045301) and Pharmalyte broad range (pH 3–10) ampholytes (P/N: 17045601) were purchased from Cytiva (Marlborough, MA).

Instrument and software: The BioPhase 8800 system (P/N: 5083590) was equipped with a UV detector from SCIEX (Framingham, MA). Data acquisition and analysis were performed using the BioPhase 8800 software, version 1.0.

JMP statistical software, version 16.2.0 was used for the design and analysis of statistical experiments.

Sample preparation: The Hemlibra sample was diluted to 5 mg/mL with deionized water before DoE method optimization. The sample, all buffers and other reagents were prepared according to instructions in the Capillary Isoelectric Focusing (cIEF) kit for the BioPhase 8800 system application guide.[1] Each sample was mixed thoroughly at room temperature. After a brief spin, a 100 µL aliquot was transferred to each well of the sample plate.

Methods and sequence creation: Methods and sequences were created using the BioPhase 8800 software. The focusing time was set between 9 and 15 minutes. The common parameters used in the separation methods include 25 kV for focusing voltage, 30 kV for a 30-min chemical mobilization and 25 psi for a 200-s injection. The cartridge and sample storage temperatures were 20°C and 10°C, respectively. Capillary conditioning and shutdown were performed as indicated in the Capillary Isoelectric Focusing (cIEF) kit for the BioPhase 8800 system application guide.[1]

Preparation of sample and reagent plates: The reagent volumes and general plate preparation followed the guidelines and recommendations found in the Capillary Isoelectric Focusing (cIEF) kit for the BioPhase 8800 system application guide.[1]

Results and discussion

Preliminary results

cIEF is a technique that provides high-resolution separation due to the amphoteric molecules in the ampholyte mixture. The electropherogram in Figure 2 shows a preliminary cIEF separation profile of Hemlibra using the broad range (pH 3-10) ampholytes and non-optimized method parameters. The profile comprises 3 distinct peak regions, each corresponding to a group of unresolved species. The main peak (MP) is located in the center, the peaks flanking the MP to the right are the acidic variants and the peaks to the left are the basic variants. These results indicate that the resolution power of the broad range ampholyte is insufficient to separate charge heterogeneity species for complex molecules, such as the bispecific mAb, Hemlibra.

Without optimization (Figure 2), the estimated pI for Hemlibra ranges from 6.6 to 6.8. The resolution between the first basic variant peak and the main peak is 1.93 and between the main peak and the first acidic variant peak is 1.82. A traditional strategy to improve resolution is to add a narrow range of ampholytes to the broad range of ampholytes. The narrow range (pH 5-8) ampholyte used in this experiment[2] still required multiple parameters to be optimized.

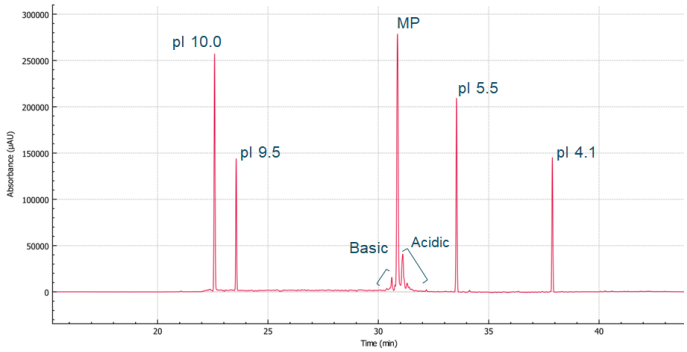

Figure 2. cIEF separation profile of Hemlibra without method optimization. MP indicates the main peak.

The design of experiments (DoE) approach

The response surface methodology (RSM) model was used for the optimization procedures. Four main factors that are known to have a significant impact on cIEF separations[2] include focusing time, the amount of narrow range (pH 5-8) ampholytes added, the amount of anodic stabilizer added and the amount of cathodic stabilizer added. Preliminary experiments (data not shown) were used to determine the lower, higher and center points (Table 1).

Table 1. Values of the 4 factors used in the DoE study.

Factors	Lower	Center point	Higher
Focusing time (min)	9	12	15
Narrow range (pH 5-8) ampholyte amount (µL)	6	8	10
Anodic stabilizer amount (µL)	6	8	10
Cathodic stabilizer amount (µL)	9	12	15

Based on the data generated using the values from Table 1, 26 runs with 2 center points were designed using the on-face Central Composite Design (CCD) software, as shown in Table 2. The 26 samples were prepared according to the values shown in Table 2.

A significant decrease in method development time was achieved by setting a sequence with a runtime of 6 hours, utilizing 8 parallel capillaries. The sequence table was arranged from small to large, according to the 9-15 minute focusing time.

Table 2. Formulation and focusing conditions for 26 samples used in the DoE study.

Sample #	Focusing time (min)	Cathodic stabilizer amount (µL)	Anodic stabilizer amount (µL)	Narrow range ampholyte (µL)	cIEF gel (µL)	Low pI marker (µL)	High pI marker (µL)	Hemlibra (µL)
1	9	9	6	6	200	2	2	8
2	9	9	6	10	200	2	2	8
3	9	9	10	6	200	2	2	8
4	9	9	10	10	200	2	2	8
5	9	12	8	8	200	2	2	8
6	9	15	6	6	200	2	2	8
7	9	15	6	10	200	2	2	8
8	9	15	10	6	200	2	2	8
9	9	15	10	10	200	2	2	8
10	12	9	8	8	200	2	2	8
11	12	12	6	8	200	2	2	8
12	12	12	8	6	200	2	2	8
13	12	12	8	8	200	2	2	8
14	12	12	8	8	200	2	2	8
15	12	12	8	10	200	2	2	8
16	12	12	10	8	200	2	2	8
17	12	15	8	8	200	2	2	8
18	15	9	6	6	200	2	2	8
19	15	9	6	10	200	2	2	8
20	15	9	10	6	200	2	2	8
21	15	9	10	10	200	2	2	8
22	15	12	8	8	200	2	2	8
23	15	15	6	6	200	2	2	8
24	15	15	6	10	200	2	2	8
25	15	15	10	6	200	2	2	8
26	15	15	10	10	200	2	2	8

Three response factors (RF) were considered for statistical analysis. The resolution between the MP and the first basic variant peak and the resolution between the MP and the first acidic variant peak were both considered. Further, the peak symmetry of the first pI marker was used to indicate the complete focusing time of the cIEF analysis. After the analysis, the effect summary revealed that the focusing time and 2 additional interactions are most important for the separation. These interactions occur between the cathodic stabilizer and the ampholytes and between the focusing time and the ampholytes.

The software provides a prediction profiler feature that can help determine the optimal condition to increase the resolution of cIEF separation for Hemlibra.

The final optimized conditions were determined from the experiment, as follows:

- 10 minute focusing time
- 15 µL of cathodic stabilizer
- 6 µL of anodic stabilizer
- 6 µL of narrow-range (pH 5-8) ampholyte
- 8 µL of sample
- 200 µL of cIEF gel with 4M urea
- 2 µL of low-pI marker
- 2 µL of high-pI marker

The cIEF profiles before and after methods optimization via DoE are shown in Figures 2 and 1, respectively. The increased resolution of the basic variant peaks highlights the power of this DoE study.

Table 3 shows the remarkable improvement in the resolution between the acidic and basic variants and the main peak. Figure 3 shows that the reproducibility achieved under the optimized conditions was equally notable and Table 4 summarizes the average values and %RSD calculated for the 56 injections.

Table 3. Improvement of resolution between the MP and basic and acidic variants.

Resolution	MP and basic	MP and acidic
Before	*1.81*	*1.93*
After	*5.07*	*3.90*

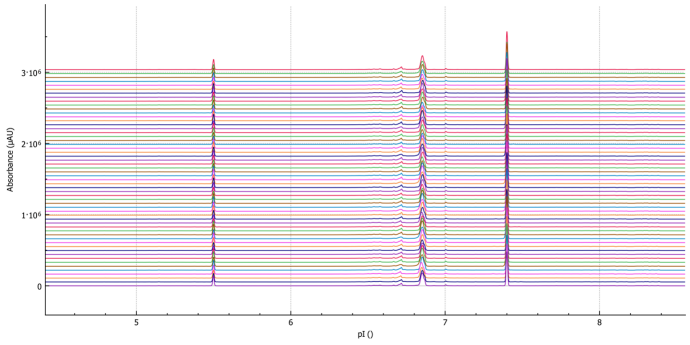

Figure 3. Reproducible separation utilizing the optimized cIEF method for Hemlibra. Overlay of cIEF separation from 56 injections.

Table 4. pI and peak area % composition of Hemlibra after methods optimization.

	Basic peaks		Main peak		Acidic peaks	
	pI	Peak area %	pI	Peak area %	pI	Peak area %
Average (n=56)	*7.00*	*2.49*	*6.85*	*80.09*	*6.72*	*17.42*
% RSD	*0.02*	*5.29*	*0.02*	*1.01*	*0.02*	*4.07*

Conclusions

- DoE approach to cIEF method development for the bispecific antibody, Hemlibra, was accelerated from days to 6 hours
- The optimized method is highly reproducible for pI and area % composition
- Significant improvement in resolution between MP and basic or acidic variants was achieved

References

1. Capillary Isoelectric Focusing (cIEF) kit for the BioPhase 8800 system application guide, RUO-IDV-05-8651-B
2. S. Mack, I. Cruzado-Park, J. Chapman, C. Ratnayake, G. Vigh, Electrophoresis 2009, 30, 4049–4058.

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