Short communication

Development of ion exchange chromatography methods for monoclonal antibodies

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1. Introduction

Monoclonal antibodies (MAbs) have been widely developed as biopharmaceutical agents to treat a number of diseases, such as asthma, arthritis, cancers, and multiple sclerosis, etc. MAbs are often found existing in multiple isoforms with different net charges. These isoforms are evident as multiple bands on isoelectric focusing (IEF) gel analysis [1]. The multiplicity of IEF bands may be due to a combination of amino and carboxyl termini heterogeneity of the heavy and light chains, the deamidation of asparagine residues, sialylation of carbohydrate moiety, and glycation of lysine residues [2]. According to International Conference on Harmonization (ICH) guidance document [3], drug substance heterogeneity defines its quality, the degree and profile should be monitored and characterized to ensure lot-to-lot consistency. The characterization of

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charge heterogeneity is therefore critical to the successful development of therapeutic antibodies. To isolate and study isoforms of proteins and monitor their distributions, many different techniques, such as slab gel electrophoresis, capillary electrophoresis (CE) [4–11], ion exchange chromatography (IEC) [12–20], and hydrophilic interaction chromatography (HIC) [21,22] are used. Compared with the other techniques, IEC has a larger selection of commercial columns and is a potential nondenaturing preparative procedure to isolate the isoforms for subsequent characterization. However, due to the large molecular size of MAbs, successful separation of isoforms of MAbs by IEC is not often seen in publications. Moorhouse et al. [12] used papain to fragment the monoclonal antibody into Fab and Fc prior to chromatographic analysis. The smaller fragments allow separation of the variants arising from certain specific sites. Kaltenbrunner et al. [13] used a linear pH gradient combined with a salt gradient to improve the separation. In this report we describe a systematic approach to develop IEC

methods for MAbs. We used high efficient exchange resin, smaller internal diameter columns, and higher flow rate to achieve fast and high degree separation.

Table 1
Column characteristics

Column (vendor)	Dimension (mm)	Particle size (μm)	Functional group	Matrix type	pH stability
Mini S PE, (Pharmacia Biotech)	4.6 × 50	3	Methyl sulfonate; strong cation exchanger	Polymer based non-porous hydrophilic matrix	1–14
BioScale S2, (Bio-Rad)	7 × 52	10	Sulfonate; strong cation exchanger	Polymer based macroporous hydrophilic matrix	1–14
PolyCAT, (PolyLC)	4.6 × 200	5	Poly aspartic acid; weak cation exchanger	Silica based porous (300 A)	2–8
IEC SP-825, (Shodex)	8 × 75	5–10	Sulfopropyl; strong cation exchange	Polyhydroxymethacrylate very polar matrix	2–12
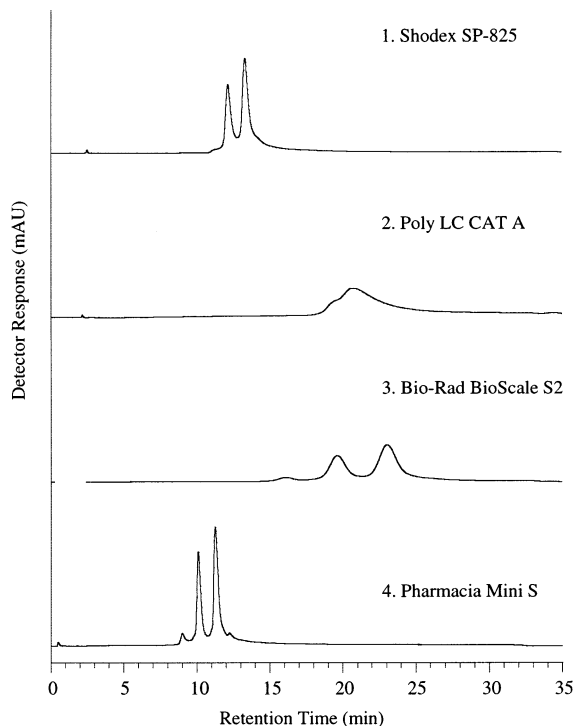
Mobile phase A: 20 mM sodium phosphate, pH 6.5, mobile phase B: mobile phase A plus 1 M NaCl, pH 6.5; Gradient: 0-30% B linear over 30 minutes; Flow rate: 1.0 ml/min; Detection: UV@ 220nm; Sample: 30 μg of IgG 1

Fig. 1. Column selectivity comparison on the separation of IgG 1.
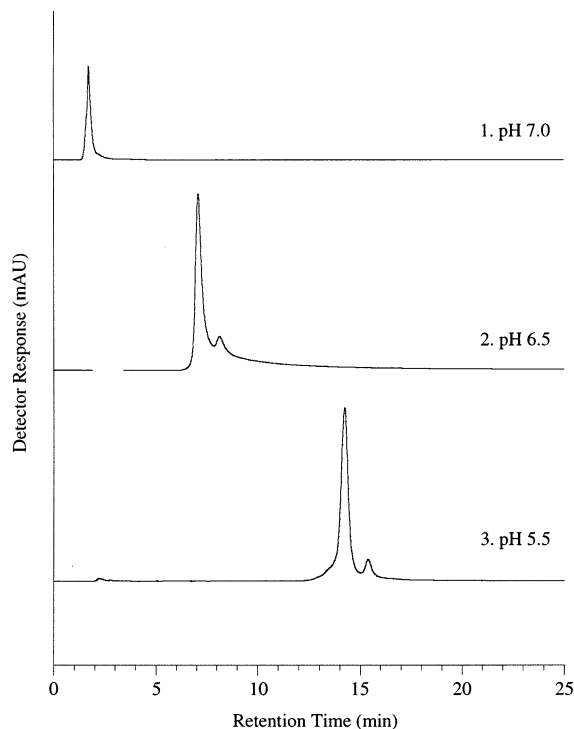
1. pH 7.0 (phosphate buffer); 2. pH 6.5 (phosphate buffer); 3. pH 5.5 (acetate buffer). Column: Shodex SP-825 ; Mobile phase A: 20 mM buffer, mobile phase B: mobile phase A plus 1 M NaCl; Gradient: 0-20% B linear over 30 minutes; Flow rate: 1.5 ml/min; Detection: UV@220 nm; Sample: 30 µg of IgG 4

Fig. 2. Impact of buffer pH on the separation of IgG 4.

2. Experimental and result discussion

All experiments were performed using an Alliance 2690 HPLC (Waters, Milford, MA) coupled with an ABI 757 UV detector (Perkin Elmer, Norwalk, CT) recording at 220 nm. Deionized water was freshly prepared using a Milli-Q Plus purification system (Millipore, Bedford, MA). All buffer and salts used were at least reagent grade. Monoclonal antibodies IgG 1 and IgG 4 were manufactured and purified by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). The chromatographic separation columns used: Mini S PE was purchased from Pharmacia Biotech (Piscataway, NJ); BioScale was purchased from Bio-Rad (Hercules, CA); PolyCAT was purchased from PolyLC (Columbia, MD); IEC SP-825 was from Shodex (Showa Denko, Japan).

### 2.1. Selection of separation mode and columns

Two monoclonal antibodies IgG 1, and IgG 4, studied were both basic proteins having major pI values of 9.3, and 8.3, respectively. To resolve these proteins in the anion mode would require a mobile phase at a pH above 10. This was not a viable separation mode because this extreme pH causes deamidation of proteins, which is one of the causes of isoform variation that was been studied. Therefore, cation mode was the obvious choice to avoid these problems. To control the cost and screening time, four cation exchange columns were screened based on previous experience and vendors' literature. Three of these were strong cation exchange columns, and the fourth one was a weak cation exchange column. The strong cation exchange resins were all polymer-

based, and all had sulfonic acid functional groups with small matrix variation such as carbon chain length, or bonding characteristics. Strong cation exchangers are able to retain their capacity at relative low pH, and have advantage of working under a wide pH range. The weak cation exchanger was silica based and contained poly aspartic acid functional groups, which are somewhat similar to the amino acids of the proteins. From each product group we selected the smallest particle size available, and a column geometry that allowed fast flow rate. The detail description of the columns is listed in Table 1. Fig. 1 shows that column selectivity is unique for monoclonal antibody IgG 1, even though the Mini S and BioScale S2 columns have very similar column chemistry. The difference of selectivity may be due to slight differences in the manufacture process, particle and pore size. The chromatographic separation performed on the Mini S

(Fig. 1) shows that IgG 1 has four peaks, and the two major peaks have achieved almost baseline separation. Comparison of the chromatograms generated by the four columns in Fig. 1, Mini S separated IgG 1 isoforms better than the other three, and had the least run time. It was therefore selected for subsequent method development of IgG 1.

2.2. Selection of mobile phase pH

To obtain sufficient binding of the proteins to the cation exchange columns, the pH was chosen at least 1 pH unit below the pI value. Usually lower pH gives more positive charge to the protein and increases retention times. Mobile phase pH was screened at the same time as the columns were screened. Firstly, a pH condition was decided to be tested, then a buffer was chosen if its pK_a value was close to that pH, and was UV
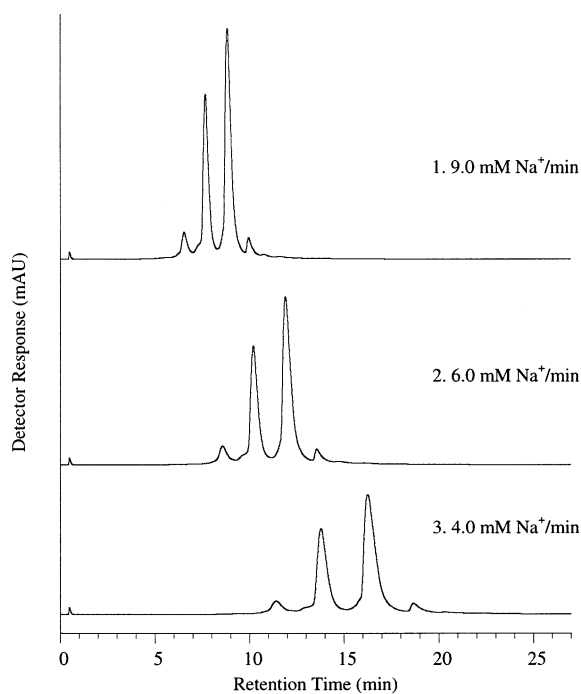
Mobile phase A: 40 mM sodium phosphate, pH 6.5, mobile phase B is A plus 0.4 M NaCl. 1. 9.0 mM Na$^+$/min or 0-45%B over 20 min.; 2. 6.0 mM Na$^+$/min or 0-45%B over 30 min.; 3. 4.0 mM Na$^+$/min or 0-30%B over 30 min; Flow rate: 1.5 ml/min; Column: Mini S; Detection: UV @ 220 nm; Sample: 75 μg of IgG 1

Fig. 3. Effect of gradient slope on retention time and peak shape.
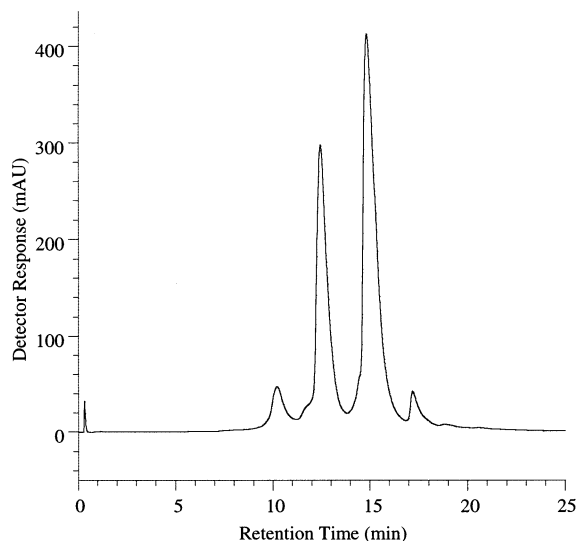
Column: Mini S PE 4.6 x 50 mm, 3 μm (Pharmacia Biotech, Cat# 17-5005-01)
Column Temperature: ambient
Detection: UV @220 nm
Flow Rate: 1.5 ml/min.
Mobile Phase: A: 40 mM sodium phosphate, pH 6.5
               B: mobile phase A plus 0.4 M NaCl, pH 6.5
Gradient: 0-25% B linear, over 25 minutes
Sample: 75 μg of IgG 1

Fig. 4. HPIEC method for separation of IgG 1.

transparent. Secondly, mobile phase A was prepared with 20 mM buffer concentration and adjusted to that pH, mobile phase B was then made by adding sodium chloride to mobile phase A to yield a final concentration of 1 M, and adjusted to the same pH. Using these buffers and a 30-min gradient, the four columns were screened to compare selectivity. After screening the columns under one pH, the process was repeated under other pH conditions. Because column functional groups are also affected by change of the buffer pH, there may not be a single pH that works best for all different columns. Finally, the pH that gave the best selectivity was chosen. Fig. 2 shows the chromatograms of IgG 4 under pH 7.0 (phosphate buffer), pH 6.5 (phosphate buffer) and pH 5.5 (acetate buffer). IgG 4 showed the best separation at pH 5.5 with the slight drawback of longer run time.

2.3. Selection of gradients

The initial salt concentration and gradient slope determine retention time and peak shape of the MAb chromatography. Fig. 3 shows the effect of three different gradient slopes on the resolution of IgG 1 using the same initial salt concentration. Initial buffer concentration, gradient slope and types (such as multi stage and non-linear gradients) often need numerous experiments to optimize. To save time, the equations below were used to predict the retention time of the peak of interest in a new experiment. In most cases, the salt concentration at retention time, $C_{\text{Salt RT}}$ (at which the peak of interest elute out), the slope of gradient, and initial salt concentration, $C_{\text{Initial salt}}$, hold an approximate equation as follows:

$$C_{\text{Salt RT}}(\text{Na}^+)$$
$$= \text{RT (min)} \times \text{Slope of gradient (Na}^+/\text{min)}$$
$$\div C_{\text{Initial Salt}}(\text{Na}^+)$$

After obtaining $C_{\text{Salt RT}}$ value from the first one or two experiments, rearrange the above equation to estimate the new retention time when change to a new gradient in the subsequent experiments:

$$\text{RT} = (C_{\text{Salt RT}} - C_{\text{Initial Salt}})$$
$$\div \text{Slope of new gradient}$$

A number of experiments showed that this relationship was a good prediction of the retention time. Additional experiments showed that the multi stage and non-linear gradients did not provide any extra benefit, a simple linear gradient program was good in most applications. After the gradient slope was optimized, the initial buffer concentration was raised while the gradient slope was maintained in order to reduce the run time and increase buffer capacity.

By applying the above approach, we were able to quickly find the major elements needed for the new IEC method. We also optimized the other minor parameters, such as flow rate, injection volume, etc. The final two separate methods developed for IgG 1 and IgG 4 are shown in Figs. 4 and 5, respectively. Although a full-scale validation has not yet been carried out, the experiment
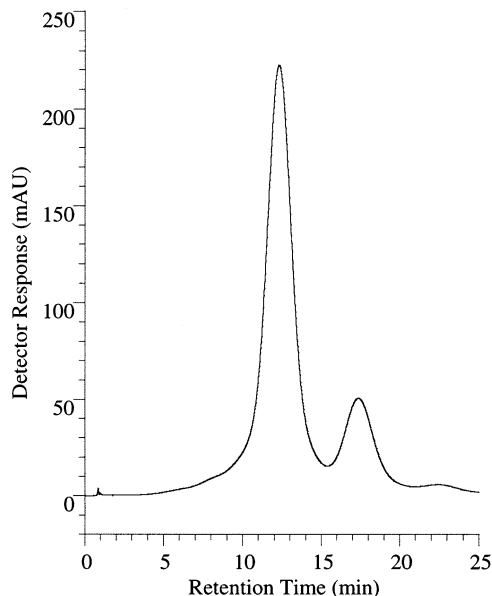
Column: BioScale S2: 7 x 52 mm, 10 μm macroporous (Bio-Rad, Cat# 751-0011)
Column Temperature: ambient
Detection: UV @220 nm
Flow Rate: 1.5 ml/min.
Mobile Phase: A: 40 mM sodium phosphate, pH 6.5
　　　　　　　B: mobile phase A plus 0.4 M NaCl, pH 6.5
Gradient: 0-15% B linear, over 25 minutes
Sample: 75 μg of IgG 4

Fig. 5. HPIEC method for separation of IgG 4.

data showed a fairly good reproducibility (R.S.D. $< 1\%$), and linearity ($R^2 > 0.99$ for a 10-fold concentration range).

3. Conclusions

- The approach presented in this report is a cost-effective way to develop a new IEC method for MAbs.
- pH is the most important experiment parameter in IEC. It has the biggest impact on the separation and should be determined first.

- Although IEC has relative low separation power comparing with reversed-phase-HPLC, it is still possible to achieve a baseline separation if a suitable column can be identified. In this study, two separate IEC methods have been successfully developed for IgG 1 and IgG 4.

Acknowledgements

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