POROS® A20 Analytical HPLC Columns for the Quantitation of Monoclonal Antibodies

Introduction

POROS® A20 columns, containing 20 µm POROS® beads functionalized with recombinant Protein-A, enable the rapid and precise quantitation of monoclonal antibodies and other Fc-containing biomolecules from sample solutions such as harvested cell culture fluid and downstream product pools. The practicality, high throughput, and robustness of assays developed using these columns has led to their widespread adoption in biopharmaceutical analytical procedures.

This application note provides recommended operating conditions that will help maximize assay performance and column lifetime. However, since antibodies and sample solutions differ widely between various applications, operating conditions should be optimized to accommodate the specific needs of each unique assay.

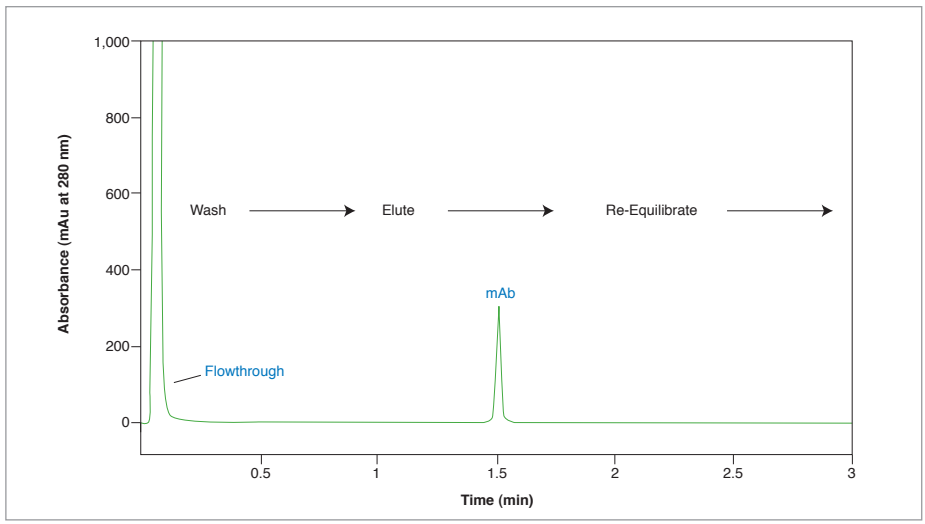

Figure 1. Typical Chromatogram for Injection of Harvested Cell Culture Fluid Containing Monoclonal Antibody (mAb). 20 µL injection, 1 mg mAb/mL.

System Setup

A pre-column filter should be utilized to protect against column fouling. A filter pore size in the range of 0.5 µm to 2 µm is recommended—for example, Upchurch Part# A-315, A-316, A-318, A-355, or A-356.

UV monitoring can be performed at 280 nm, or for increased sensitivity, 214 nm.

Samples can be assayed at room temperature. If numerous samples are being loaded in a sequence, autosampler temperature control should be set at 2–8°C.

Sample Preparation/Injection

Standards and samples should be 0.2 µm filtered prior to loading in sample vials, as centrifugation of samples may not be sufficient to ensure clarity. Bubbles residing at the bottom of a sample vial should be shaken free.

Recommended injection volume for standards and samples is between 20 µL and 100 µL. Injection volume should be equivalent for all standards and samples, and should not exceed the column bed volume. Sample injection volumes can be increased if samples are too dilute. Highly concentrated samples can be diluted with equilibration buffer prior to injection.

Since the A20 assay is based on recombinant Protein-A affinity, most sample solution components will not interfere with binding or

elution. Common excipients that should not interfere include: ⩽1% Tween 20, ⩽1% Tween 80, <1% Triton X-100, <20% polysaccharides, and <20% glycols. If desired, the addition of 10 mM EDTA can help with the chelation of ferric compounds that often exist in cell culture samples and can result in nonspecific binding of impurities to the A20 column.

Figure 1 shows a typical chromatogram for a 20 µL injection of harvested cell culture fluid containing monoclonal antibody at 1 mg mAb/mL.

Standard Curve

Standard curves should be generated from purified material, and unique standards should be used for each molecule being assayed. Standard curve data are typically fit by linear regression. Linear correlation coefficients and assay precision are typically excellent (R^2 >0.98, coefficient of variation <10%). Dynamic range will be dependent in part on instrumentation (e.g., UV detector linearity at high antibody concentration).

Figure 2 shows standard curves generated with purified monoclonal IgG. Table 1 outlines method conditions used.

Buffer Preparation

A simple two-buffer system is typically used for column operation. A typical equilibration/wash buffer (Buffer A) is 25–100 mM phosphate, pH 6.6–7.5, with or without sodium chloride up to 150 mM. A typical elution buffer (Buffer B) is the same buffer at pH 2.0–3.5. Other elution buffer components that may be used include hydrochloric acid, glycine, citrate, acetate, or other components that buffer well at low pH.

Preparing the elution buffer with a concentration equal to or greater than that of the equilibration buffer will help ensure a good pH transition during elution. Preparing the elution buffer with a salt concentration equal to the equilibration/wash buffer will minimize shifts in the UV baseline during elution that are particularly evident when UV monitoring is performed at 214 nm and when analyzing dilute samples. The characteristics of the baselines achieved during equilibration and elution can be assessed by running blank samples. Figure 3 shows the influence of elution buffer composition on peak height, peak width, and retention time. Table 1 outlines the method conditions used.

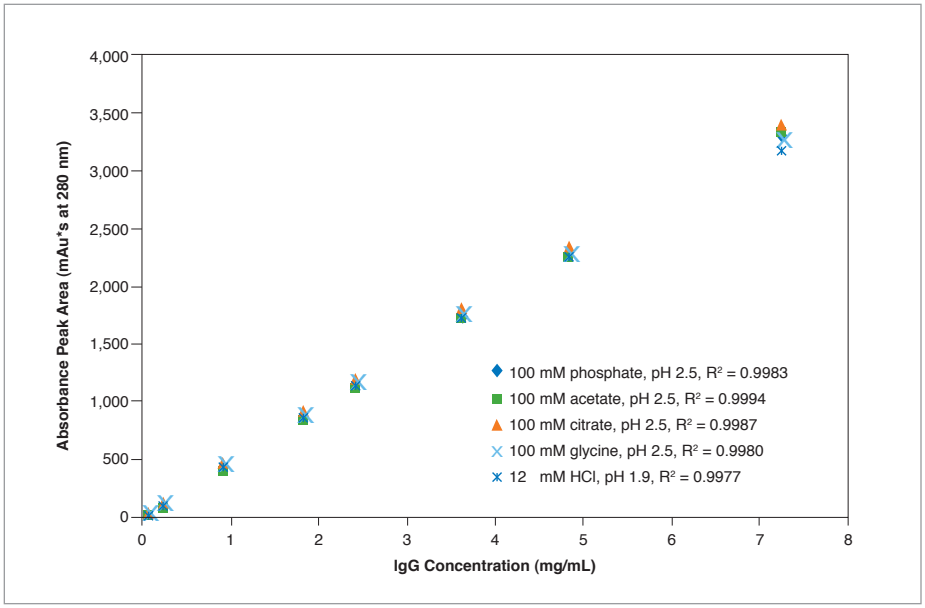

Figure 2. Standard Curves for Monoclonal IgG Eluted With Various Elution Buffers. Data points are mean values of replicate samples (maximum CV = 3.5%). Method conditions are listed in Table 1.

Table 1. Method Conditions Used for Figures 2 and 3.

Column:	POROS® A20 columns, 2.1 mmD x 30 mmH, 104 µL Part# 2-1001-00 (PEEK) or 1-5024-12 (stainless steel)		
System:	Agilent 1200 HPLC		
Flow:	3.0 mL/min, 5,200 cm/hr		
Injections:	20 µL purified monoclonal IgG, 0.1 to 7.2 mg/mL		
UV Detection:	280 nm		
Equilibration/Wash Buffer A:	50 mM phosphate, 150 mM NaCl, pH 7.0		
Elution Buffer B, one of:	100 mM citrate, pH 2.5 100 mM acetate, pH 2.5 100 mM phosphate, pH 2.5 100 mM glycine, pH 2.5 12 mM HCl, pH 1.9		
Method Timetable:	Time (min)	Gradient (%B)	
	0.00	0	Equilibration/Wash, 0.5 min, 14 CV
	0.50	0	
	0.51	100	Elution, 1.0 min, 29 CV
	1.5	100	
	1.51	0	Reequilibration, 1.5 min, 43 CV
	3.00	0	

NOTE: *It is important that complete elution is attained. If only partial elution is attained, then antibody will remain bound to the column between injections, column fouling will begin, and carryover may affect results. The completeness of elution is assessed by recovery of the standards.*

Method Timetable and Flow Rate

In order to ensure effective pH transition, elution should consist of a step to 100% Buffer B. Elution should not consist of a gradient or a blend of Buffer A and Buffer B that results in less than 100% Buffer B being used for elution.

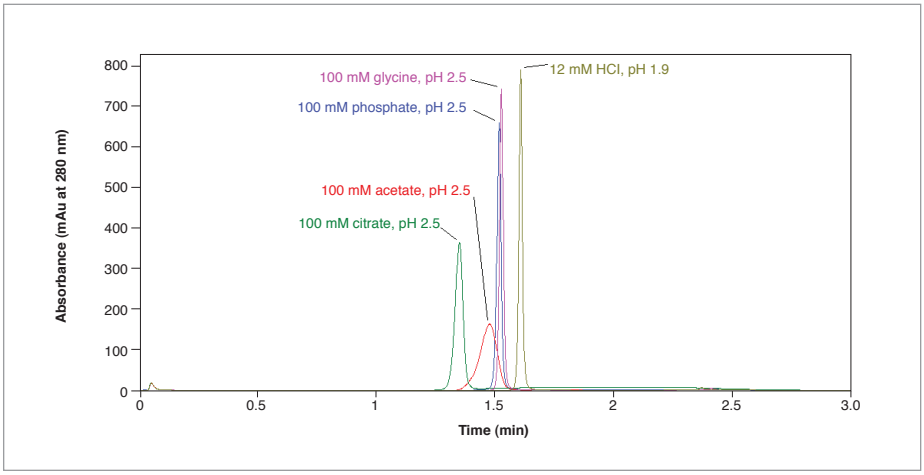

Figure 3. Chromatograms for Monoclonal IgG Standard Eluted With Various Elution Buffers. 20 µL injection, 1.8 mg mAb/mL. Method conditions are listed in Table 1.

The duration of wash, elution, and equilibration stages should provide sufficient column volumes of flow to allow for complete pH transitions and establishment of UV baselines.

Flow rate can range from 800 to 8,600 cm/hr. Columns are packed at 180 bar, and thus operating pressure limits are high. To ensure bed stability, columns should not be operated over 180 bar.

Column Reuse and Cleaning

Columns are generally very robust; lifetimes of 3,000 injections per column have been reported. Extended reuse should be accompanied by monitoring of column backpressure and an assay control sample.

If backpressure increases or control sample recovery changes, the column should be cleaned to remove residual material from the column frits and from the POROS® A20 media.

Typical cleaning solutions include 2–6 M guanidine hydrochloride, 1 M acetic acid, 20% ethanol, 1 M acetic acid plus 20% ethanol, 20% isopropanol, elution buffer titrated to pH 1.5–2.0, and elution buffer plus 1–2 M sodium chloride.

A cleaning cycle should involve 2 or 3 injections of cleaning solution at a volume equal to the column bed volume, followed by 2 or 3 injections of equilibration buffer—for example, 2 x 100 µL cleaning solution, 2 x 100 µL equilibration buffer. Alternatively, multiple column volumes of a desired solution can be run over the column. If desired, the cleaning can be run in reverse flow to help clean the top frit, with flow direction returned to normal after cleaning. If the system does not allow for flow reversal, the column can be plumbed in reverse, cleaned, and returned to normal after cleaning. The cleaning sequence should be monitored for removed protein peaks.

Conclusions

The flexibility and robustness of POROS® A20 columns makes the selection of suitable operating conditions a straightforward process. Fine-tuning of assay parameters within the broad guidelines outlined above is encouraged to facilitate optimized results and to enable maximum resource efficiency for each unique analytical application. When properly used, POROS® A20 columns enable a long lifetime of rapid, accurate quantitation of monoclonal antibodies.

Scientific Contributors

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ORDERING INFORMATION

Description	Size	Column Material	Part Number
POROS A20 Analytical HPLC Column	2.1 mmD x 30 mmH	PEEK	2-1001-00
POROS A20 Analytical HPLC Column	2.1 mmD x 30 mmH	Stainless Steel	1-5024-12

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