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Direct determination of residual Pluronic F-68 in in-process samples from monoclonal antibody preparations by high performance liquid chromatography

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ABSTRACT

A simple and sensitive high performance liquid chromatography (HPLC) method was developed to determine residual Pluronic F-68 (PF-68) in in-process samples of monoclonal antibody (MAb) preparations. The method permits the direct injection of proteinaceous samples after simple sample dilution and is able to quantitate as low as 50 mg/L of PF-68 in the presence of up to approximately 30 g/L of protein. The PF-68 molecule was separated on a restricted access reversed phase column using a step gradient and then measured by an evaporative light scattering detector (ELSD). The method was successfully applied to demonstrate PF-68 clearance in MAb purification processes. A modified colorimetric method using liquid–liquid extraction and cobalt thiocyanate to derivatize PF-68 is also described. The results obtained by both the HPLC and colorimetric methods were compared. In addition to its ease of use and simplicity, the HPLC method had better accuracy and higher throughput than the colorimetric method.

1. Introduction

PF-68 is a low-foaming, non-ionic surfactant that has multiple functional effects on mammalian cells in cell cultures. High concentrations of PF-68 increase the resistance of cells to shear forces in hydrodynamic culture situations such as shaker and spinner cultures [1,2]. In large-scale mammalian cultures, PF-68 protects cells from the detrimental effects of bubble sparging for oxygenation [3]. The protection provided by PF-68 is due to its ability to reduce cell to bubble attachments [4], to form a stable foam layer [5] and to decrease plasma membrane fluidity by adsorption to and incorporation into the cell membranes [6]. Pluronic also prolongs the survival of cells exposed to chemical stresses such as mineral starvation or excess, or to physical stresses such as mechanical stress or hyperthermia [7]. Therefore, PF-68 is widely used in large scale mammalian cell cultures to produce therapeutic proteins. However, because PF-68 is considered to be a process-related impurity [8], its clearance during the purification processes of therapeutic proteins should be demonstrated. The objective of the work described in this paper was to develop an easy and accurate HPLC method for quantitation of PF-68 in proteinaceous in-process samples to support process development of therapeutic monoclonal antibodies.

PF-68 is one of the non-ionic triblock copolymers known as Poloximers, also known by the trade name Pluronics. It is comprised of block polymers in which a central polypropylene oxide group is flanked by two polyethylene oxide groups:

$$HO(CH_2CH_2O)_{75}(CHCH_3CH_2O)_{30}(CH_2CH_2O)_{75}H$$

PF-68 has an average molecular weight of 8400. Because this single-bonded molecule lacks a UV chromophore, derivatization is usually necessary for quantitative determination. Several colorimetric methods have been developed to quantitate certain Pluronics [9–17]. Based on the type of derivatizing reagent used, they can be grouped into the cobalt thiocyanate method [9–12], the ammonium ferrothiocyanate method [13,14], the iodine method [15], the potassium tetrakis (4-halophenyl) borate method [16], and the tetraiodobismuthate method [17]. These methods were developed for the quantitation of nonionic surfactants in water and samples containing no or very little proteins. They cannot be applied directly to the determination of PF-68 in in-process samples from MAb purification processes without the use of protein removal procedures. With the advent of high-titer MAb production processes, in-process samples that require PF-68 testing will have higher protein concentrations than before and this trend is anticipated to continue. The increasing protein concentrations in samples create an additional challenge to the method accuracy and precision as the limits of the protein removal are reached and residual proteins interfere with quantitation.

Various chromatographic techniques, including normal phase, reversed phase, size exclusion, and ion exchange, have been reported for the determination of non-ionic surfactants [18]. More recently, a method for quantitation of Pluronics in a pharmaceutical formulation was developed using size exclusion chromatography

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(SEC) [12]. This SEC method provided satisfactory sensitivity and a wide linear range. However, approaches for dealing with proteinaceous samples were not addressed in these methods. Similar to the colorimetric methods, these chromatographic methods will also require sample preparations to remove proteins, which otherwise will result in column fouling. Therefore, it would be highly desirable if a method can directly analyze proteinaceous samples and use a small sample volume without sacrificing precision and accuracy.

Restricted access media (RAM) materials have been developed for the analysis of small molecules in biological matrices by direct injections [19,20]. This paper describes the development of a HPLC-RAM method that overcomes the aforementioned difficulties for quantitation of PF-68 in MAb in-process samples.

2. Experimental

2.1. Reagents and materials

Acetonitrile, methanol, ethyl acetate and acetone were purchased from Burdick & Jackson (Muskegon, MI, USA), acetic acid from EMD (Gibbstown, NJ, USA), trichloroacetic acid (TCA) from Sigma (St. Louis, MO, USA), and ammonium thiocyanate, cobalt (II) nitrate hexahydrate and PF-68 from Fluka (Milwaukee, WI, USA). Water was purified with a Milli-Q Filtration System (Millipore, Billerica, MA, USA) with a minimum 18 MΩ cm resistivity.

2.2. HPLC-RAM-ELSD method

2.2.1. Standard and sample preparation

A 2000 mg/L PF-68 stock solution was prepared by weighing 0.2 g of PF-68 into a 100-mL volumetric flask, and the material was completely dissolved in a 0.1% acetic acid solution by stirring on a stir plate. A 100 mg/L PF-68 solution was made by diluting the 2000 mg/L stock solution with 0.1% acetic acid solution. A set of standard solutions consisting of 10, 25, 50, 75 and 100 mg/L were then prepared from the 100 mg/L PF-68 solution using the 0.1% acetic acid solution as a diluent. Because of the nonlinear response property of ELSD, a quadratic equation was used to fit the standard curve.

All samples were diluted a minimum of 5-fold with the 0.1% acetic acid solution to avoid sample carryover. Additional dilutions for high PF-68 samples were made so that the PF-68 level in the diluted samples would fall into the middle range of the standard curve. As a result, all measured results were multiplied by a dilution factor of at least 5 to obtain the final PF-68 concentrations in tested samples. All the samples were tested with the finalized method as described in Section 2.2.3.

Four different types of MAbs were employed in the method development and performance evaluation. The MAb purification sequence started from centrifugation, filtration, Column A, and Column B, and ended with Column C. The Column A Load sample was obtained after the filtration. The Column A, B and C Pool samples were taken after Columns A, B and C, respectively. All the four MAbs used the same type of Column A, but had different types of Columns B and C. The buffers used for the column conditioning, sample loading and sample elution were not identical among the four MAbs.

In the method qualification, only two types of the MAb 1 in-process samples were selected for demonstration purposes: MAb 1 Column A Load sample with a PF-68 level above the limit of quantitation (LOQ) of 50 mg/L (which is referred to as the sample LOQ due to 5-fold dilution of tested samples) and MAb 1 Column C Pool sample with a PF-68 level below the sample LOQ. For the method linearity, repeatability, accuracy, and LOQ evaluations, the Column A Load sample was diluted to a PF-68 level close to the method LOQ (which is 10 mg/L), and the Column C Pool sample was diluted 5-fold. Five PF-68 levels (10, 25, 50, 75 and 90 mg/L with 3 replicates each) were spiked into both of the samples, respectively, as part of the sample dilution. Unspiked samples were also prepared in triplicate. For intermediate precision evaluation, the Column A Load sample was diluted to a PF-68 level close to the middle range of the standard curve, while the Column C Pool sample was diluted 5-fold and a 50 mg/L PF-68 standard was spiked to the sample as part of the sample dilution. To further evaluate its applicability, the finalized HPLC method was employed to test all the four in-process samples of the four MAbs.

2.2.2. Chromatographic system

The chromatographic system consisted of an Agilent 1100 HPLC system (Palo Alto, CA, USA) equipped with a pump, autosampler, a temperature-controlled column compartment with a switching valve, and a SEDEX Model 85 evaporative light scattering detector (Alfortville Cedex, France). The analytical column used for separation was a Cadenza HS-C18 column (150 mm × 3.0 mm, 3 μm particle size) purchased from Imtakt Corporation (Philadelphia, PA, USA). The ELSD settings were as follows: nebulizer temperature at 50 °C, gas pressure between 3.3 and 3.6 bar, gain at 7, and unit/volt at 5,000,000. Nitrogen gas was supplied by an in-house nitrogen gas generator.

During the method development, four types of columns were evaluated: Acclaim Surfactant (4.6 mm × 150 mm, 5 μm) purchased from Dionex (Sunnyvale, CA, USA), Hi-Sep (2.1 mm × 150 mm, 5 μm) from Supelco (St. Louis, MO, USA), AV-2 (4.6 mm × 150 mm, 5 μm) from GL Sciences (Shinjuku-Ku, Tokyo, Japan) and Cadenza HS C18.

2.2.3. PF-68 separation conditions

PF-68 was separated on the Cadenza HS C18 with mobile phases consisting of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The gradient program used in the final method is shown in Panel G3 of the insert in Fig. 1. The flow rate was 0.4 mL/min. The sample injection volume was 15 μL and a 20% acetonitrile solution was used to wash the needle before each sample injection. The total run time was 35 min. To prevent ELSD from contamination by proteins, a post-column switching valve was employed to divert the flow from 0 to 5 min to the waste line. The flow was then switched back to the ELSD detector from 5.1 to 35 min. Autosampler temperature was set at 4 °C, but the column temperature was not controlled.

2.3. Colorimetric method

For the purpose of comparison, PF-68 was also analyzed with a colorimetric method. The colorimetric method is based on the formation of a dark blue complex between PF-68 and cobalt thiocyanate. The absorbance of the dark blue complex was measured at 624 nm. A cobalt-thiocyanate reagent solution was prepared by dissolving 20.0 g of ammonium thiocyanate and 3.0 g of cobalt nitrate hexahydrate in 100 mL of water.

2.3.1. Standard and sample preparation

A 1500 mg/L PF-68 stock solution was prepared by dissolving 0.15 g of PF-68 in 100 mL of water with stirring on a stir plate. A set of standards consisting of 50, 100, 500, 1000, and 1500 mg/L PF-68 were then made by diluting the stock solution with water. In-process samples with a PF-68 level of greater than the highest standard were diluted with water to fall within the linear range of the calibration curve, and no dilution was made for all other samples. The method LOQ of the colorimetric method was 50 mg/L.

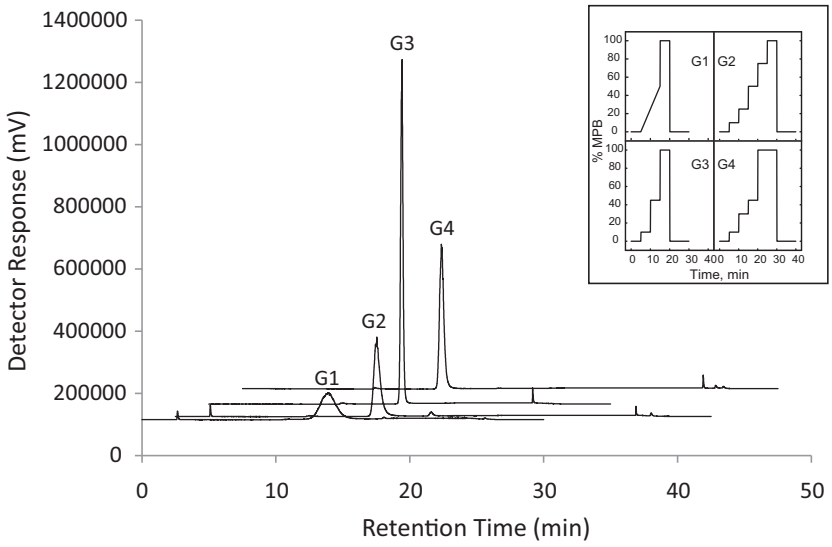

Fig. 1. Chromatograms of 100 mg/L PF-68 separated by four types of gradients. The gradients are shown in the insert, and G1–G4 represent Gradient 1–Gradient 4, respectively.

For samples with a PF-68 level below the method LOQ, dilution was not required, therefore the sample LOQ was also 50 mg/L.

2.3.2. Colorimetric assay procedure

The procedure was modified from the cobalt thiocyanate method [11] by adding a protein removal step. To a 1.5-mL microcentrifuge tube, 250 μL of standard or sample, 25 μL of TCA and 250 μL of methanol were added. The capped tube was vortexed for 0.5 min and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a separate microcentrifuge tube containing 250 μL of cobalt-thiocyanate reagent and 250 μL of ethyl acetate. The sample was mixed for approximately 0.5 min on a vortex mixer and centrifuged at 12,000 rpm for 5 min. The upper two layers were removed using a vacuum aspiration device. The pellet and tube wall were washed three times with 1 mL of ethyl acetate. The washed sample was air-dried with the tube open in a fume hood for more than 15 min. One microliter of acetone was added to dissolve the dried pellet and the tube capped immediately. The tube was vortexed until the pellet was dissolved completely. The absorbance was measured at 624 nm on a Cary 50 Bio UV-Vis Spectrophotometer from Varian (Walnut Creek, CA, USA).

3. Results and discussion

3.1. HPLC-RAM-ELSD method development

3.1.1. Column selection

To select a suitable analytical column, four different types of columns were evaluated in terms of PF-68 separation, protein accumulation in the columns, and separation reproducibility by different column lots. Acclaim Surfactant, Hi-SEP and AV-2 columns were evaluated with the mobile phases of 100 mM ammonium acetate at pH 5.4 (A) and acetonitrile (B) and the flow rates were 1.0, 0.2 and 1.0 mL/min, respectively. The mobile phases used to evaluate the Cadenza HS-C18 column were 0.1% acetic acid (A) and acetonitrile (B) and the flow rate was 0.4 mL/min. PF-68 was well separated by the Acclaim Surfactant column, but the accumulation of proteins in the column was observed. No PF-68 separation was obtained on the Hi-SEP column. The AV-2 column had the capability to separate PF-68, but showed poor lot-to-lot reproducibility. The Cadenza HS-C18 column not only provided the best separation and lot-to-lot reproducibility, but also was able to exclude proteins. Therefore, the Cadenza HS-C18 column was chosen for further evaluation.

3.1.2. Mobile phase gradient selection

Proteins elute in the void volume on the Cadenza HS C18 column because the hydrophilic groups on the outer surfaces of the column packing material prevents protein from entering the inner surfaces. Since an organic solvent might cause protein precipitation, an aqueous mobile phase A was employed to elute proteins. After the elution of proteins, a mobile phase containing an organic solvent must be applied to elute analytes of interest. Initially, various linear gradients were examined, but peak shape and height were not ideal. Next, different step gradients except Gradient 1 (G1) were evaluated with the 0.1% acetic acid as mobile phase A and acetonitrile in the 0.1% acetic acid as mobile phase B. The mobile phase B levels of the four gradients are shown in the insert of Fig. 1 and the corresponding chromatograms are shown in Fig. 1. It could be seen that the gradients had a profound effect on the PF-68 signal. Gradient 3 (G3) produced the narrowest peak width and the highest peak height compared to the other gradients. Therefore, G3 was selected for the final PF-68 separation gradient of the method. In the very early method development, protein was monitored, but was switched to a waste line in the final assay. A representative chromatogram of protein containing samples without use of a switching valve is shown in Fig. 2 using the G3 gradient.

3.1.3. Elimination of carryover

It was observed that the area of the PF-68 peak increased continuously after repeated injections of undiluted samples (50 μL), indicating a possible carryover from the previous injection. Modifying the mobile phase gradient and reducing the sample injection volume to 15 μL and 3 μL did not improve the situation. Up to three water blank injections following a 3 μL sample injection still did not eliminate the carryover peak. To alleviate the carryover effect, three types of the MAb 1 in-process samples were diluted 3-, 4- and 5-fold, respectively, with a 0.1% acetic acid diluent. Each of the diluted samples (15 μL) was injected followed by a water blank injection. The peak area at the PF-68 retention time in the water blank chromatogram was used as a measurement of the carryover. The carryover was observable in the 3-fold and 4-fold diluted samples (except one in 4-fold dilution), but not in the 5-fold diluted samples (Table 1). This indicated that the acidic

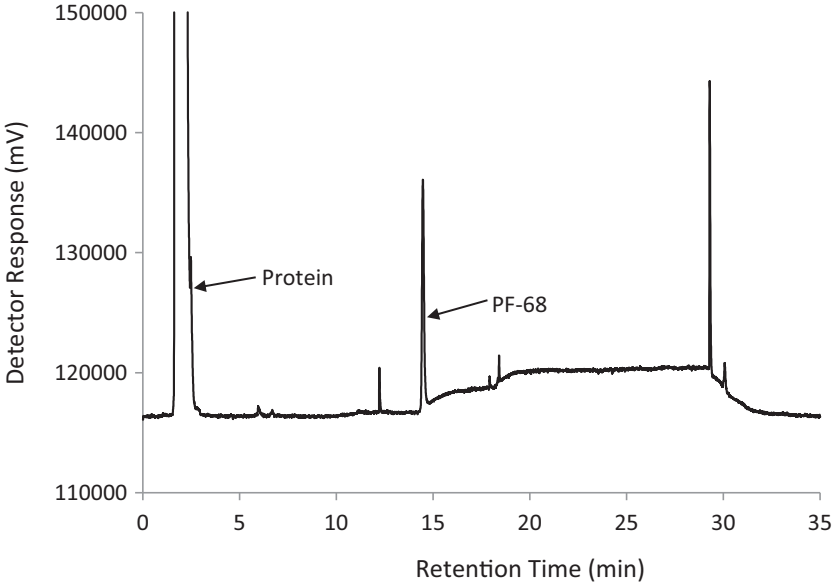

Fig. 2. Protein eluted at the void volume. Because a switch valve was used, the protein peak did not show on the chromatogram of the final assay procedure.

dilution played a critical role in eliminating the carryover. After implementing a 15-μL injection of the 5-fold diluted samples, no carryover was further observed in the routine multiple sample analyses.

3.1.4. Determination of the standard curve range

The method LOQ was determined to be 10 mg/L using a signal to noise ratio approach [21]. Since a clearance assay mainly focuses on analyte concentrations less than or close to the LOQ level, a relatively narrow range of standard concentrations from 10 to 100 mg/L PF-68 was evaluated. Within this range, the PF-68 response curve was not linear (Fig. 3). A non-linear response curve on an ELSD has been reported for many analytes [22]. A quadratic equation was found to be the best fit for the standard curve range. The coefficients of determination obtained from all the standard curves tested were between 0.992 and 1.000.

3.1.5. Spike recovery calculation

Due to the non-linear nature of the ELSD response curve, it was observed that spike recoveries, especially at the low end of the standard curve, were affected significantly by PF-68 levels present in a diluted sample. When the peak area of PF-68 in a diluted (at least 5-fold) sample was within the range of the standard curve, spike recoveries could be calculated by a common approach, Approach 1 shown below. However, when the PF-68 peak area in a diluted (5-fold) sample was less than that of the 10 mg/L standard, the spike recovery determined by Approach 1 was incorrect. To determine an appropriate approach, six different types of MAb samples with PF-68 levels below the sample LOQ were evaluated. The samples were diluted 5-fold, and four PF-68 levels were spiked into each sample type as part of the sample dilution. The spiked samples were tested and the recoveries were calculated using the following three approaches:

Approach 1, $R_s = (C_m$ in spiked sample $- C_m$ in unspiked sample$)/C_s \times 100\%$
Approach 2, $R_s = (C_m$ in spiked sample $- 0)/C_s \times 100\%$
Approach 3, C_r was calculated first from the standard curve using a corrected peak area, i.e., the difference in the peak area between spiked sample and unspiked sample, and R_s was then calculated as follows:

$$R_s = C_r/C_s \times 100\%$$

where R_s is the spike recovery, C_m is the measured concentration, C_s is the spiked concentration and C_r is the recovered concentration.

The results calculated by the three approaches are shown in Table 2. All the spike recoveries obtained from Approach 1 were below 100% with a decreasing trend from the 90 mg/L spike to the 10 mg/L spike. With Approach 3, the spike recoveries were between 100% and 109% without a significant trend related to the spike levels. The spike recovery results calculated by Approach 2 were not as good as those by Approach 3. Furthermore, the relative standard deviation (RSD) value of the spike recoveries from 24 samples obtained by Approach 3 was the smallest. Therefore,

Table 1
Effect of sample dilutions on carryover.

Sample dilution	Area of the carryover peak		
	MAb 1 Column A Pool sample	MAb 1 Column B Pool sample	MAb 1 Column C Pool sample
3-Fold	18,239	11,832	6,431
4-Fold	9,978	7,783	0
5-Fold	0	0	0

Table 2
Spike recoveries calculated by three different approaches.

PF-68 spike level	Number of samples[a]	Average spike recovery		
		Approach 1	Approach 2	Approach 3
10 mg/L	6	48%	114%	109%
25 mg/L	6	79%	105%	102%
50 mg/L	6	88%	101%	100%
90 mg/L	6	95%	102%	102%
Overall average	24	77%	106%	103%
Overall RSD		24%	6.2%	4.5%

[a] The PF-68 level in these six diluted samples was below 10 mg/L.

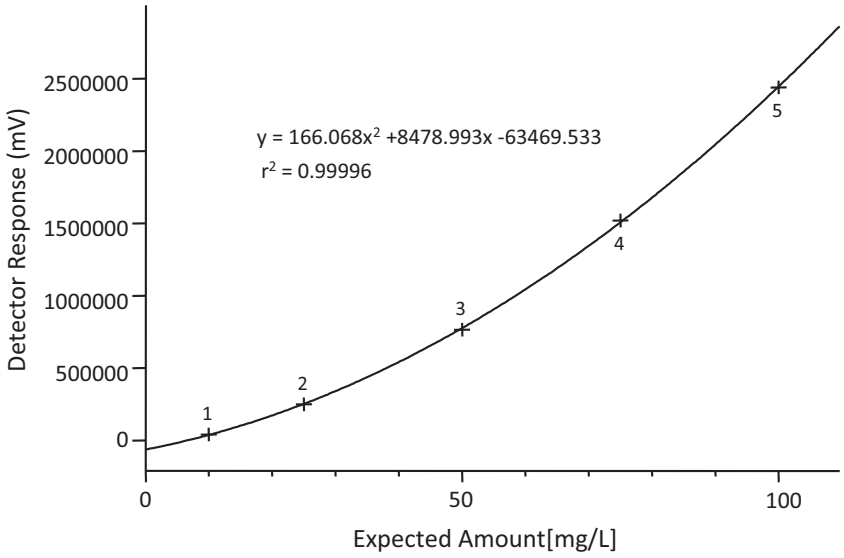

Fig. 3. A typical standard curve for PF-68.

Approach 3 was the best approach to calculate the spike recoveries for diluted (5-fold) samples with PF-68 levels below the method LOQ of 10 mg/L.

3.2. HPLC-RAM-ELSD method qualification

The method performance parameters including specificity, linearity, accuracy, repeatability, intermediate precision, and limit of quantitation were evaluated using two types of MAb 1 samples: the Column A Load sample and the Column C Pool sample.

Specificity. The method specificity was examined by comparing chromatograms of the Column A Load sample and its corresponding cell culture medium, and by comparing chromatograms of the Column C Pool sample with and without PF-68 spiking. No significant interference with PF-68 determination in the sample matrices was found (Fig. 4).

To verify if other common surfactants interfere with the PF-68 determination, polypropylene glycol, polysorbate 20, polysorbate 80 and Triton X-100 were prepared at the 50 mg/L concentration and subjected to the assay. None of these surfactants co-eluted with the PF-68 peak (data not shown), which demonstrated that the present method would not have an interference issue if one of these compounds is used in the purification processes.

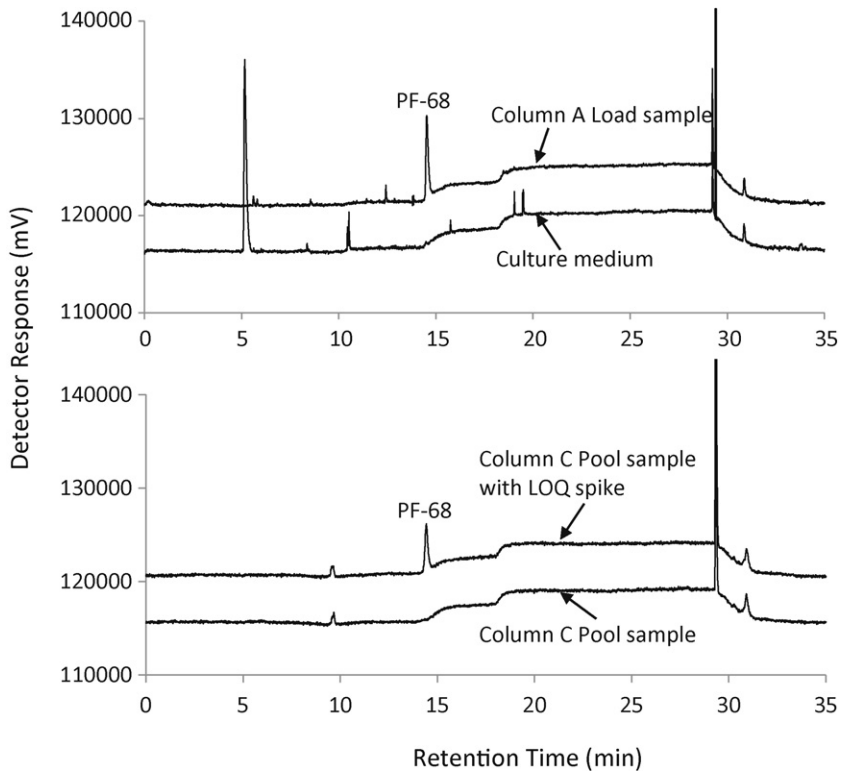

Fig. 4. Specificity evaluation of the method.

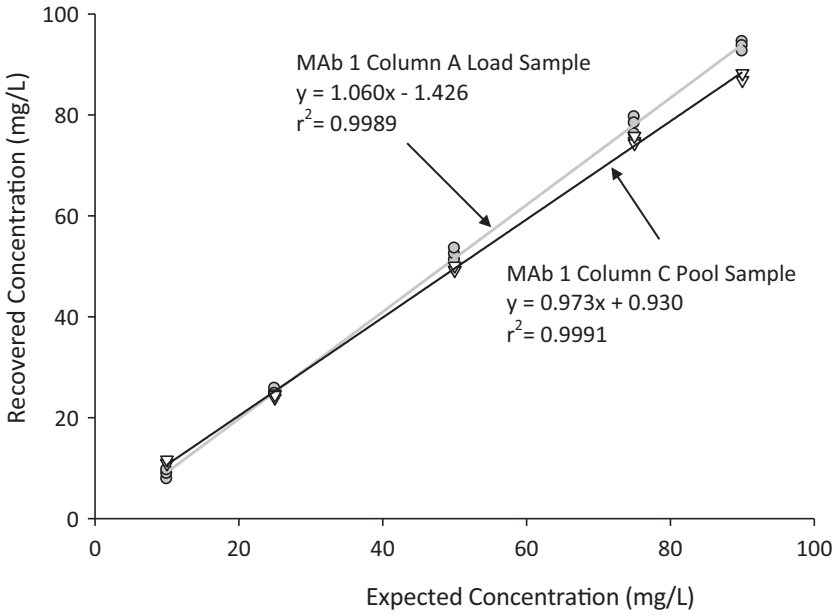

Fig. 5. The linearity plot of recovered vs. expected concentrations for MAb 1 Column A load sample (▽) and MAb 1 Column C pool sample (●).

Table 3
Repeatability and intermediate precision.

Sample	RSD (n = 15)	
	Repeatability[a]	Intermediate precision[b]
MAb 1 Column A Load	5.4%	3.4%
MAb 1 Column C Pool	5.8%	5.9%

[a] The RSD value for the repeatability determination was calculated from 15 spike recoveries in each sample type using the data in Fig. 5.
[b] The RSD value for the intermediate precision evaluation was calculated from 15 measured PF-68 concentrations on the same sample over 5 days with 2 analysts, 2 instruments, and 2 columns.

Linearity. Because of the non-linear response, linearity was further evaluated by determining the correlation between the recovered and expected concentrations of the PF-68 standards with a range of 10–90 mg/L spiked into the Column A Pool and Column C Pool samples, respectively. The recovered concentration in the Column A Load sample was calculated by subtracting the PF-68 concentration in the unspiked samples from that in each of the spiked samples, while the recovered concentration in the Column C Pool sample was calculated as described in Approach 3 in Section 3.1.5. The coefficient of determination (r^2) for the regression equation of recovered vs. expected concentrations was 0.9989 for the Column A Load sample and 0.9991 for the Column C Pool sample (Fig. 5).

Accuracy. Using the data shown in Fig. 5, the method accuracy was calculated from the slope of the regression equation of the recovered vs. expected PF-68 concentrations, multiplying by 100%. The accuracy was 106% for the Column A Load sample and 97% for the Column C Pool sample.

Table 4
Accuracy and precision at the method LOQ.

Sample	Accuracy[a]	Precision[a]
MAb 1 Column A Load	92%	4.1%
MAb 1 Column C Pool	112%	3.7%

[a] Accuracy and precision at the method LOQ were measured by the average and RSD values, respectively, of three spike recoveries, which were calculated from Fig. 5, at the 10 mg/L spike level.

Table 5
PF-68 concentrations (mean ± standard deviation) in four MAb preparations determined by the HPLC method. PF-68 clearance in the three column pool samples was demonstrated in all four monoclonal antibodies.

Sample type	Monoclonal antibody[a]			
	MAb 1 (n[b] = 4)	MAb 2 (n = 3)	MAb 3 (n = 3)	MAb 4 (n = 3)
Column A Load	719 ± 31	658 ± 21	682 ± 30	813 ± 19
Column A Pool	<50[c]	<50	<50	<50
Column B Pool	<50	<50	<50	<50
Column C Pool	<50	<50	<50	<50

[a] Four MAbs used the same type of Column A, but different types of Columns B and C.
[b] n is the number of lots tested.
[c] The method LOQ of the HPLC method was 10 mg/L and the sample LOQ was 50 mg/L due to 5-fold dilution.

Repeatability and intermediate precision. The repeatability was determined by using the RSD value of 15 spike recoveries in each sample type calculated from Fig. 5. The RSD values were ≤5.8% for both sample types (Table 3). The intermediate precision was evaluated on the same type of the samples over 5 days with 2 analysts, 2 instruments, and 2 columns. The RSD values of the 15 testing results for both sample types were ≤5.9% (Table 3).

Limit of quantitation. To determine if the method was able to quantitate PF-68 at the LOQ level in each diluted sample type, the recoveries at the 10 mg/L spike level (n = 3) were calculated using the data from Fig. 5. The average and the RSD value of these recoveries were used as accuracy and precision measurements, respectively. The accuracy was 92% for the Column A Load sam-

Table 6
Accuracy and precision of colorimetric and HPLC methods. The values were calculated from the spike recovery studies of six sample types with 3 spike levels covering the standard curve range for each method. A total of 18 spike recovery levels were prepared in triplicate. The overall averaged recovery and overall averaged RSD from the 18 spike recovery levels were used as accuracy and precision measurements, respectively.

	Colorimetric method	HPLC method
Accuracy	96.6%	100.6%
Precision	6.3%	2.6%

Table 7
PF-68 concentrations in in-process samples of MAb preparations determined by the two methods.

Monoclonal antibody	Sample type	No. of lots	PF-68 (mean ± SD) (mg/L)		% difference[a]
			Colorimetric method	HPLC method	
MAb 1	Column A Load	4	615 ± 30	719 ± 31	17%
MAb 1	Column A Pool	4	<50[b]	<50[c]	NM[d]
MAb 1	Column B Pool	4	<50	<50	NM
MAb 1	Column C Pool	4	<50	<50	NM
MAb 2	Column A Load	3	580 ± 14	658 ± 21	13%
MAb 3	Column A Load	3	590 ± 14	682 ± 30	16%

[a] % difference = ((concentration from HPLC − concentration from colorimetric)/concentration from colorimetric) × 100%.
[b] The method and sample LOQs of the colorimetric method were 50 mg/L.
[c] The method LOQ of the HPLC method was 10 mg/L and the sample LOQ was 50 mg/L due to 5-fold dilution.
[d] NM, not meaningful.

ple and 112% for the Column C Pool sample, and the precision was ≤4.1% for both sample types (Table 4). The acceptable accuracy and precision results confirmed that the method LOQ of 10 mg/L PF-68 was valid for both sample types.

In summary, the acceptable qualification results indicated the method capability to measure PF-68 in the proteinaceous in-process samples of MAb preparations.

3.3. Determination of PF-68 in in-process samples of different monoclonal antibodies

The HPLC method was further evaluated for its applicability to testing in-process samples of three other monoclonal antibodies in addition to MAb 1. Because different MAb types were purified with different upstream processes, different columns, and different buffers, the sample matrices among the four MAbs were expected to vary significantly. However, no significant interference was observed during the method qualifications for all the sample types, and all the qualification results were within the ranges shown in Section 3.2. The PF-68 concentrations in all these in-process samples of the four MAbs are shown in Table 5. In the Column A Load samples, the PF-68 concentrations were between 658 and 813 mg/L, which were close to the expected values of the corresponding MAbs. The PF-68 levels were below the sample LOQ (50 mg/L) in all the column pool samples. The results confirmed that the HPLC method has good accuracy and specificity, and can provide wide applications to quantitation of PF-68 in different types of sample matrices from different MAb preparations. Furthermore, it was shown that the first purification column (i.e., Column A) was able to remove PF-68 to below the sample LOQ level. Thereafter, PF-68 remained consistently at or below the sample LOQ level. The results demonstrate the capability of the MAb purification processes to consistently remove PF-68.

3.4. Comparison of HPLC method and colorimetric method

For comparison, the recoveries of PF-68 standards covering the standard curve range spiked into six sample types with a total of 18 spike levels in triplicate at each level for each method were compiled. The average recovery and RSD at each spike level ($n = 3$) were first obtained. The overall averaged recovery was then calculated from 18 average recoveries and the overall averaged RSD from 18 RSDs. The overall averaged recovery and averaged RSD were used as measurements of the accuracy and precision, respectively. The results are shown in Table 6. The accuracy for the HPLC method was 100.6%, which was better than that for the colorimetric methods. Similar to the accuracy, the precision of the HPLC method was also better than that of the colorimetric method. Furthermore, three Column A Load samples from three different MAbs and three column pool samples of the same MAb were tested by both methods. For the Column A Load samples, the average concentrations determined by the HPLC method were 13–17% higher than those by the colorimetric method (Table 7). The HPLC results were closer to the expected values than the colorimetric results, confirming the HPLC method had better accuracy. Relative to the HPLC method, the lower PF-68 levels obtained by the colorimetric method might be due to the loss of some PF-68 during the long sample preparation process. However, the precision was comparable between two methods. For the three column pool samples, all the PF-68 levels were <50 mg/L from both methods (incidentally the methods share the same sample LOQ). The comparable results obtained from both methods further support the above conclusion that clearance of PF-68 occurred following the first purification column. In addition, both methods were able to handle in-process samples with protein concentrations of up to approximately 30 g/L.

With respect to the method execution, the colorimetric method is time-consuming and labor intensive, since it involves multiple steps from sample cleanup, to derivatization of PF-68 and to absorbance measurements, and the entire procedure has to be executed manually. The manual preparation also demands meticulous sample handling by the analyst. The HPLC method is far easier to implement, and the only sample preparation required is sample dilution. With an autosampler and software, sample testing and data processing can be performed automatically. As a result, the HPLC method can minimize assay errors and increase sample throughput. Therefore, the HPLC method will be more suitable and applicable for determining PF-68 concentrations and clearance in in-process samples to support the process development of therapeutic monoclonal antibodies.

4. Conclusion

An HPLC method for direct determination of PF-68 in the proteinaceous in-process samples of MAb preparations was developed using a RAM column and ELSD detection. The method performance results demonstrated that the method had acceptable specificity (no matrix interference), linearity ($r^2 > 0.998$), repeatability (≤5.8% RSD) and intermediate precision (≤5.9% RSD), and accuracy (97–106%), and that it was sensitive (method LOQ = 10 mg/L). A colorimetric method was also adopted by modifying a pre-existing cobalt thiocyanate method. Both the HPLC and colorimetric methods were suitable for quantifying PF-68 levels in in-process samples and demonstrating PF-68 clearance in MAb productions. Samples with high proteins (up to approximately 30 g/L) were successfully analyzed by both methods. Of the two methods, the HPLC method is simpler and easier to use, and has better accuracy and higher throughput.

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