Determination of Pluronic F-68 in High Protein Matrices by HPLC-RAM-ELSD

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Abstract

An HPLC method was developed to determine Pluronic F-68 (PF-68) in in-process samples from monoclonal antibody (MAb) preparations. The method uses a Cadenza HS-C18 column, a restricted access media (RAM) stationary phase, that permits the direct injection of proteinaceous samples after a single sample dilution. The HPLC system consisted of a pump, an autosampler, a column switching valve, and an evaporative light scattering detector (ELSD). PF-68 was separated with two mobile phases, 0.1% acetic acid (HAc) and 0.1% HAc in acetonitrile, with step gradient elution. Proteins eluted in the void volume and were diverted into a waste line, while PF-68 eluted at 14.5 min. A quadratic equation was used to fit the standard curve. The present method offers the advantages of simple sample preparation (a single dilution) and sensitivity (10 mg/L quantitation limit).

Introduction

PF-68 is a low-foaming, non-ionic surfactant that is widely used in mammalian cell culture processes. Because PF-68 is not a generally recognized as safe reagent, its clearance during the purification processes should be demonstrated. PF-68 is often determined by a colorimetric method. This type of method requires tedious sample preparation procedures to isolate the PF-68 from the proteins present in the samples. However, the Cadenza HS-C18 column has the capability to exclude proteins while separating small molecules. The column is a hybrid octadecylsilyl stationary phase that has hydrophilic groups on the outer surface and hydrophobic groups within the pores. A sample with a high protein concentration can be injected directly on the column without a clean-up procedure. The objective of this study was to develop an HPLC method with this column for determination of PF-68 in samples with high protein concentrations.

Materials and Methods

HPLC System:	Agilent 1100 pump and autosampler
Detection:	ELSD
Column:	Cadenza HS-C18 (150 x 3.0mm, 3µ particle size)
Mobile Phase:	A: 0.1% HAc B: 0.1% HAc in acetonitrile
MP Gradient:	0 – 5.0 min, 0%B; 5.1 – 10.0 min, 10%B;10.1-15.0 min 45%B; 15.1-25 min 100%B; 25.1-35 min 0%B
Col. Temp.:	Ambient
Injection Vol.:	15 µL
Flow Rate:	0.4 mL/min
Runtime:	35 min
Sampler Temp.:	4ºC
Dilution Soln.:	0.1% HAc

Results and Discussion

Column Selection

Three types of protein exclusion columns were evaluated. The Cadenza HS-C18 column was found to be suitable for separation of PF-68 (Table 1).

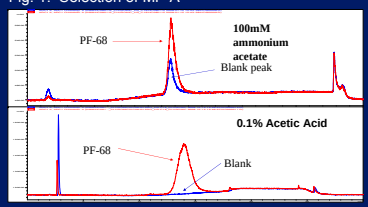
Table 1. Columns for PF-68 separation

Column Type	Hi SEP	AV-2	Cadenza HS-C18
Runtime	30 min	30 min	35 min
Flow Rate	0.2 mL/min	1.0 mL/min	0.4 mL/min
Observation	No separation	Poor reproducibility	Good separation
Selection	No	No	Yes

Mobile Phase Selection

For Mobile Phase (MP) A, 100 mM ammonium acetate and 0.1% HAc were examined. A blank peak interfering with the PF-68 peak was seen with ammonium acetate, but not with 0.1% HAc (Fig. 1).

Fig. 1. Selection of MP A



Two HAc levels (0.1% and 0.5%) with ACN in MP B were also evaluated for PF-68 separation. A minor peak interfering with the PF-68 peak was found with 0.5% HAc, but not with 0.1% HAc (data not shown). Therefore, 0.1% HAc and ACN in 0.1% HAc were used for MP A and MP B, respectively.

MP Gradient Selection

Several gradients were used to separate PF-68. It was found that the gradients had a profound effect on the PF-68 signal. Chromatograms of four gradients are shown in Figure 2. Gradient 3 was selected (see the gradient in Materials and Methods Section).

Fig. 2. Effect of MP gradients on PF-68 signal

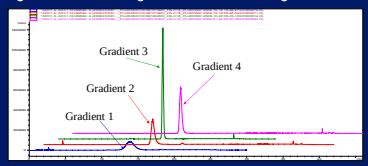


Table 2. Effect of the HAc level in the dilution solution

HAc Level	PF-68, mg/L	
	Sample A, 20x Dil.	Sample B, 5x Dil.
0.1%	33	< LOQ
1%	33	< LOQ
2%	32	< LOQ

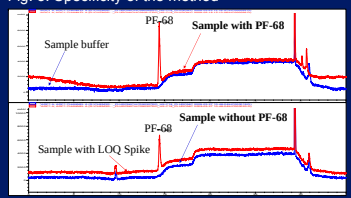
Effect of HAc Level in Dilution Solution

HAc was used in the sample dilution solution to dissociate PF-68 from proteins. Three acid levels (0.1, 1 and 2%) were examined. Samples were diluted ≥ 5x. Different acid levels did not affect the determination of PF-68 (Table 2). Since both of the mobile phases contained 0.1% HAc, this concentration was selected for a better comparability.

Method Characterization

Specificity. The method specificity was examined by comparing chromatograms of the sample and its buffer without PF-68 for the PF-68 containing sample, or chromatograms of the sample with and without PF-68 spike for the sample without PF-68 (Fig. 3). No significant interference from the two sample matrices was found.

Fig. 3. Specificity of the method



Response Curve, Range and Linearity. The responses of the standards from 10 to 100 mg/L were fitted to a quadratic equation. All the r^2 values of the tested standard curves were > 0.995. A typical standard curve is shown in Fig. 4. Linearity was evaluated by determining the correlation between the recovered and expected concentration for the PF-68 standards spiked into the tested samples (5 levels with 3 replicates each). The r^2 for Samples A and B was > 0.998 (Fig. 5).

Fig. 4. A quadratic standard curve

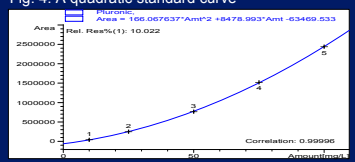
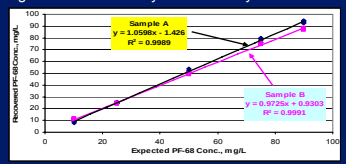


Fig. 5. Method linearity and accuracy

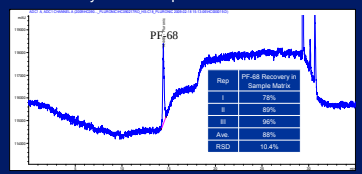


Accuracy. Using the data shown in Fig. 5, the method accuracy was calculated from the slope of the regression * 100%. The accuracy was 106% for Sample A and 97% for Sample B.

Repeatability and Intermediate Precision (IP). The RSD of the spike recoveries (n=15) calculated from Fig. 5 was used for the repeatability determination. The precision was 7.7% for Sample A and 4.5% for Sample B. The IP was evaluated by 2 analysts, 2 instruments, 2 columns over 5 days. The RSD (n=15) was 5.9%. The results indicated acceptable precision.

Limit of Quantitation (LOQ). The LOQ value was determined to be around 10 mg/L using a signal to noise ratio (10:1) approach (Fig. 6). For verification, the PF-68 standard was spiked into a sample to a final concentration of 10 mg/L and the spiked sample was measured. The precision and accuracy at the LOQ level are shown in the insert of Fig. 6. Good accuracy and precision results confirmed that the LOQ was 10 mg/L.

Fig. 6. The 10 mg/L PF-68 signal and its precision and accuracy in the sample matrix



Robustness. DOE with two factors was used to evaluate the method robustness (Table 3). The statistical analysis showed that small variations in the compositions of the MP and column temperature did not affect the analytical results (P>0.05), indicating the robustness of the method.

Table 3. The method robustness evaluation

Factor	Composition			PF-68, mg/L			P-value
	-1	0	1	-1	0	1	
MP A-HAc	0.09%	0.10%	0.11%	33.5	33.3	33.5	0.28
Col. Temp	20C	25C	30C	33.5	33.3	33.5	0.88

PF-68 clearance in purification processes

The results show that PF-68 is cleared to below the LOQ by column A (Table 4).

Table 4. PF-68 Conc. at different column pools

In-process	Col. A Load	Col. A	Col. B	UFDF
PF-68, mg/L	690	< LOQ	< LOQ	< LOQ

Conclusion

A simple and sensitive method for determination of PF-68 was developed using the HS-C18 column and ELSD detection.. The results of specificity, linearity, precision (repeatability and IP), accuracy, LOQ, and robustness showed that the method was suitable for testing PF-68 clearance in MAb preparations.