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Application Note

Analysis of therapeutic monoclonal antibody Trastuzumab using BIOshell™ A400 Protein C4 column

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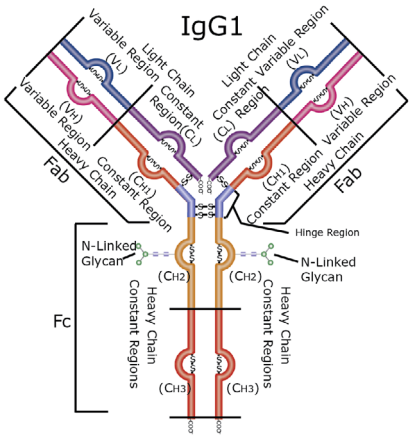
Biopharmaceuticals

Fast and high-resolution analysis of intact monoclonal antibodies (mAbs).



Abstract

Although the majority of small molecules analysed by reverse phase have a mass below 1500Da, there is a growing need to improve the performance of HPLC columns for the separation of therapeutic proteins and protein drug conjugates. This application note demonstrates a fast, high-resolution and reproducible reverse phase method for the analysis of intact therapeutic monoclonal antibody, Trastuzumab. Separation and quantification were achieved using BIOshell™ A400 Protein C4 column in less than 5 minutes, and more importantly, the optimised method was able to monitor degraded product created by heat stress studies.

Introduction

Over the past few years, monoclonal antibodies (mAbs) have become the best-selling drugs in the pharmaceutical market, and in 2018, eight of the top 10 best-selling drugs worldwide were biologics. The global therapeutic monoclonal antibody market was valued at approximately $115 billion in 2018 growing up to $300 billion by 2025. As of December 2019, 79 therapeutic mAbs have been approved by the US FDA for sales worldwide, but still there is significant potential[1]. HPLC is a well-established method for the analysis of intact mAbs by Size Exclusion and Ion Exchange chromatography. However, technological advancements in the field of Reverse Phase (RP) have made them promising tool for the analysis on intact proteins[2]. Intact mAbs are analysed with limited success using wide pore, fully porous particles due to their large size, limited diversity, long analysis time and broad peaks, compromising resolution. In contrast, high efficiency Fused-Core® columns easily separate intact mAbs quickly and with high efficiency.

Here, we have demonstrated the suitability of the BIOshell™ A400 Protein C4 column for a fast and high-resolution separation of intact Trastuzumab using RP-HPLC. Retention time and area precision of the method were excellent, demonstrating the suitability of the column. Further we also showcase quantification and robustness that is highly suitable for biopharma QC applications.

Equipments and instruments

- Shimadzu LC-2010CHT HPLC System
- Water bath
- Milli-Q water system

MERCK

Reagents, samples and materials

Cat.No.	Product Description
Materials	
1.14291	LiChrosolv® Acetonitirile Isocratic Grade
302031	Trifluoroacetic Acid HPLC grade
115333	LiChrosolv Water for chromatography
Purchased from local pharmacy	Therapeutic Trastuzumab
66825-U	BIOshell™ A400 Protein C4, 3.4 µm HPLC Column

Reagents, samples and materials

Reverse Phase HPLC Parameters

Chromatographic parameters for intact Trastuzumab using BIOshell™ A400 Protein C4 column are shown in **Table 1**.

Table 1. Chromatographic parameters used for RP HPLC analysis of Trastuzumab

Parameters	RP HPLC (intact analysis of Trastuzumab)		
Reagents, samples and materials			
Mobile Phase	A: Water + 0.1% TFA B: Acetonitrile + 0.09% TFA		
Column	BIOshell™ A400 Protein C4, 3.4 µm HPLC Column (p/n 66825-U)		
Flow rate	1 mL/min		
Injection volume	10 µL		
Trastuzumab Concentration	1 mg/mL		
Gradient Program	**Time**	**%A**	**%B**
	0	95	5
	1	95	5
	2	80	20
	6	50	50
	8	5	95
	8.1	95	5
Post Time	2 minutes		
Autosampler Temperature	5 °C		
Thermostat Column Temperature	80 °C		
Detection	280 nm		
Peak width	20 Hz		

Linearity, Limit of quantitation (LOQ) and limit of detection (LOD):

The calibration curve was constructed with nine standard concentrations of Trastuzumab from 1 to 25 µg/mL. Trastuzumab was used for LOD and LOQ measurements. The mAb concentration that provided a signal-to-noise ratio (S/N) > 3 was considered as LOD and S/N > 10 was considered as LOQ.

Forced Degradation Studies:

We compared the chromatographic profiles of native and heat-stressed trastuzumab for monitoring degraded products. For the forced degradation studies, 1 mg/mL of Trastuzumab was exposed to 10 ppm hydrogen peroxide (H2O2) followed by heating at 80 °C for 60 min. An aliquot of 10 µL was used for RP HPLC analysis.

Results and Discussions

Intact Trastuzumab Analysis

The BIOshell™ A400 Protein C4, 3.4 µm HPLC Column with Fused-Core® particles and 400 Å wide pores delivers reproducible, fast and high-resolution separation of intact Trastuzumab for biopharma development and QC applications. **Figure 1** demonstrates excellent separation and overlays of six replicates in less than 5 minutes under the chromatographic conditions.

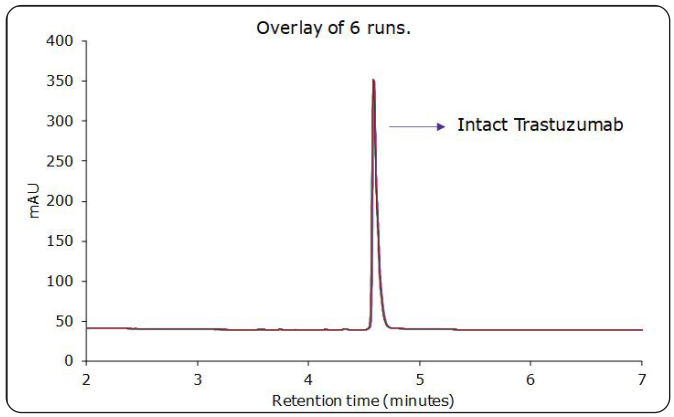


Figure 1. RP-HPLC analysis of Trastuzumab separated on BIOshell™ A400 Protein C4, 3.4 µm HPLC column.

Precision of retention time and area

Table 2 shows the average Retention Time (RT) and Area RSDs from six replicates of trastuzumab injections. The Retention Time and Peak Area RSDs were less than 0.1% and 0.29 %, respectively, which demonstrates excellent reproducibility of the method and, thus, the precision of the method.

Table 2: Retention time and peak area precision (n=6).

Sample	Retention Time		Peak Area	
	Mean (min)	RSD (%)	Mean (min)	RSD (%)
Trastuzumab (1mg/mL)	4.58	0.1	**987268**	**0.29**

Limit of detection and limit of quantitation

The LOD and LOQ were 0.125 µg/mL and 0.25 µg/mL, respectively, for Trastuzumab, indicating that the method was sensitive. Observed LOD and LOQ values of Trastuzumab are reported in **Table 3**, and the overlay of LOD and LOQ chromatograms with blank is shown in **Figure 2.**

Table .3 LOD, LOQ, and mean Area and RT (n=3)

Concentration (µg/mL)	Area	Retention Time (min)
0.125 (LOD)	9562	4.58
0.25 (LOQ)	21977	4.58

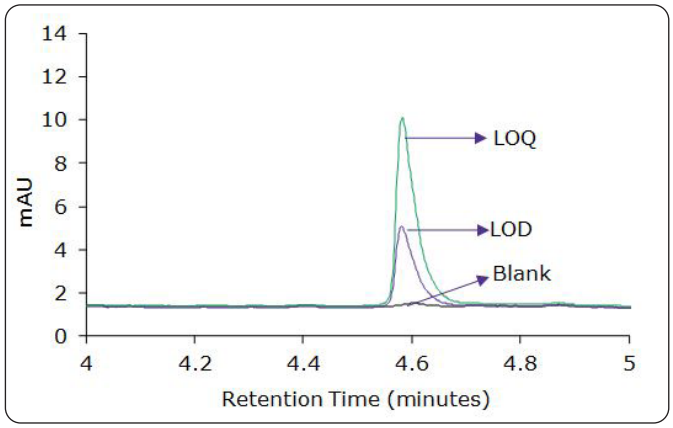


Figure 2. LOD and LOQ chromatograms of trastuzumab overlaid with blank.

Linearity

Linearity curves for Trastuzumab were constructed from 1 µg/mL up to 25 µg/mL in this study using area response and concentration of trastuzumab. The accuracy results are shown in **Table 4.** The linearity curve for trastuzumab is shown in **Figure 3.**

Table 4: Summary of linearity range (n = 3) for Trastuzumab

Trastuzumab Concentration (µg/mL)	Average Area
1	95961
2	194821
4	394886
6	593986
8	791940
10	984370
15	1480051
20	1940216
25	2447554
0.25 (LOQ)	21977

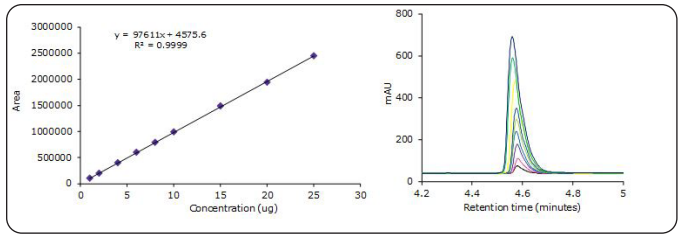


Figure 3. Linearity curve with nine standard concentrations of trastuzumab ranging from 1 to 25 µg/mL showing excellent coefficient values. Also shown are chromatogram overlays for the linearity ranges.

Trastuzumab degradation studies

We compared the intact and stressed trastuzumab using RP-HPLC to evaluate if this method is stability indicating. Any deviations in peak RT or Area as a result of stress were considered degradation products. **Figure 4** compares the RP-HPLC profile on unstressed and heat stressed trastuzumab. The profiles indicate that the BIOshell™ A400 Protein C4, 3.4 µm HPLC column was able to distinguish between unstressed and stressed trastuzumab based on the peak shape and area.

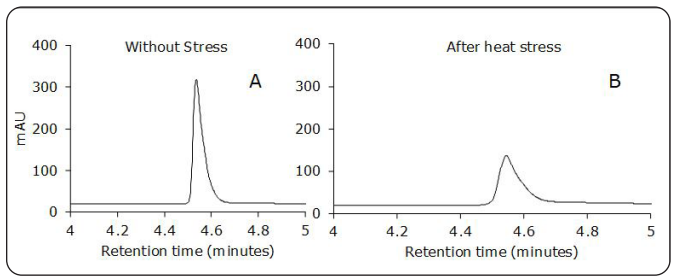


Figure 4. BIOshell™ A400 Protein C4, 3.4µm RP-HPLC profiles of unstressed (A) and heat stressed Trastuzumab sample (B).

Conclusion

Analysis of intact mAbs provides a first level of interrogation of size, post translational modification and heterogeneity. RP-HPLC analysis of mAbs requires large pore sizes, a hydrophobic stationary phase and appropriate chromatographic methods. In this application note, we showcase a simple LC-UV method for the analysis of intact trastuzumab. We used a BIOshell™ A400 Protein C4 column to develop a high resolution and rapid separation of intact trastuzumab. Area and RT precision of the method were excellent and showed the reliability of the method. Linearity curves with nine standard concentrations of Trastuzumab had excellent coefficient of linearity values indicating that the method was quantitative and accurate. The LOD and LOQ for Trastuzumab were found to be 0.125 µg/mL and 0.25 µg/mL, respectively, indicating the method was sensitive. In addition, heat stressed studies demonstrated that the BIOshell™ A400 Protein C4 column was able to monitor degraded mAbs and the method could be used for stability studies.

References

1. Han-Chung Wu et al., Journal of Biomedical Science, 27,1(2020)
2. Naves N; et al., Anal Bioanal Chem, 2013, 405, pp 9351-9363

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