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Application Note

Protein Quantification in Formulation Buffer Using a BioResolve RP mAb Polyphenyl Column

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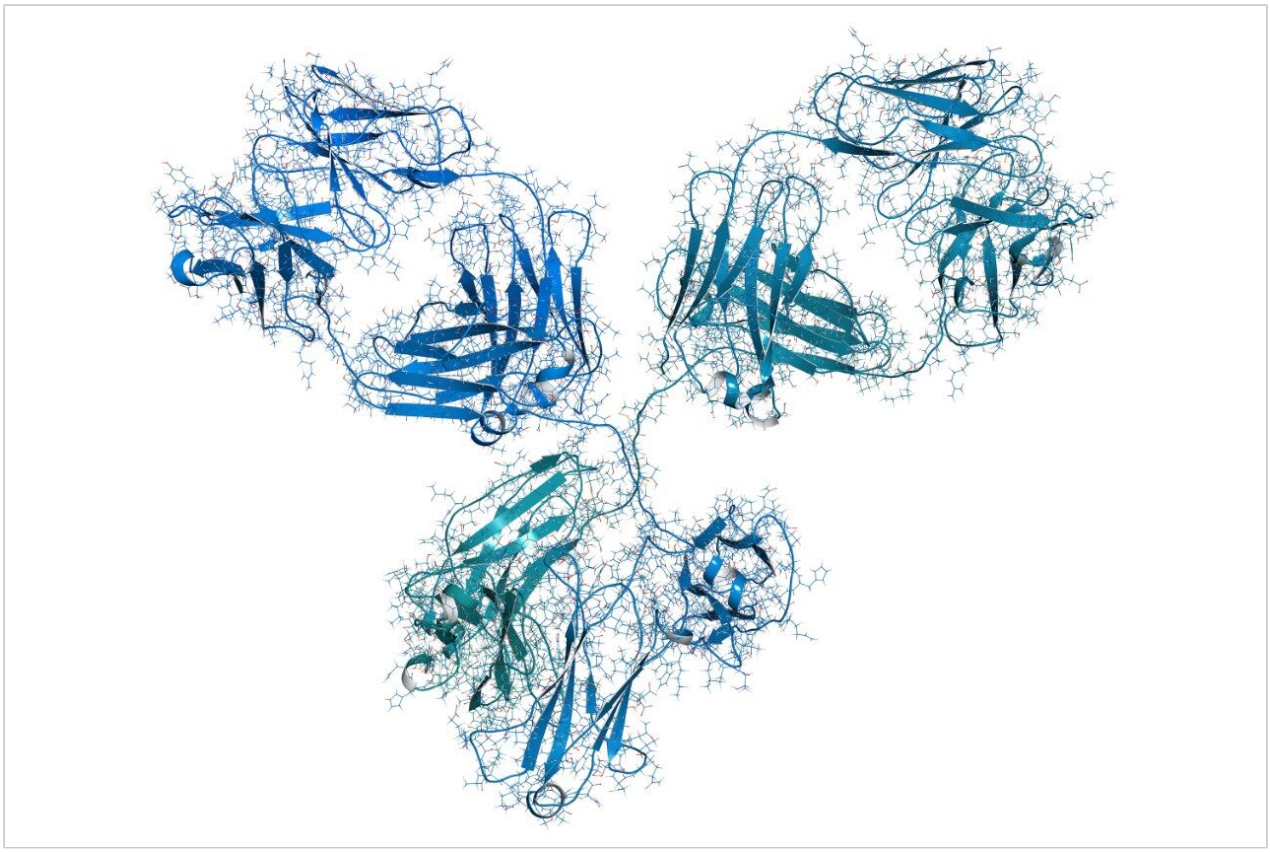

Abstract

In this application note, it is shown that the BioResolve RP mAb Polyphenyl Column, when paired with simple 0.1% TFA waters/acetonitrile mobile phases, works well as a tool to use in the quantification of various types of proteins that might be formulated in buffers containing L-histidine and rHSA.

Benefits

- Simple, fast separation of proteins from a common protein excipient, recombinant human serum albumin (rHSA)
- Excellent linearity and low % error for protein concentration determination
- Low injection-to-injection sample carryover

Introduction

Protein quantification is a basic requirement in protein drug substance and product testing. Typically, this quantification is performed using UV absorbance at 280 nm. However, there may be conditions where components of the protein formulation buffer interfere with using UV absorbance.

In this application note, a BioResolve RP mAb Polyphenyl Column is used to perform reversed-phase LC separations and to quantify rituximab (a monoclonal antibody), interleukin-12, erythropoietin, and abatacept (a fusion protein) in the presence of histidine buffer and recombinant human serum albumin (rHSA). The BioResolve RP mAb Polyphenyl Column is well suited to this application being that it is based on an efficient, silica-based, solid-core, 2.7 µm particle with an optimized 450Å pore diameter and novel polyphenyl bonding.[1,2]

BioResolve RP mAb Polyphenyl Columns and mAb Subunit Standards.

Experimental

Sample description

Rituximab, interleukin-12, erythropoietin, and abatacept were diluted into a series of concentrations using 26 mM histidine buffer, pH 6. A constant concentration (1 or 2.5 mg/mL) of recombinant human serum albumin (rHSA) was also added to each sample.

LC conditions

System:	ACQUITY UPLC H-Class Bio
System configuration:	From auto sampler to column inlet:
	Active pre-heater (APH), MP35N, 12.5 (p/n: 205000756)
	From column outlet to TUV:

	Assy, tubing inlet 0.0025 ID LT PEEK NUT, 8.5" (p/n: 700009971)
Sample temp.:	4 °C
Column temp.:	60 °C for IL-12, EPO and abatacept 80 °C for rituximab
Flow rate:	0.3 mL/min 0.2 mL/min
Injection volume:	10 µL
Column:	BioResolve RP mAb Polyphenyl, 450Å, 2.7 µm, 2.1 x 50 mm (p/n: 176004156 that contains column and intact mAb and subunit reference standards)
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, 280 nm
Sample collection/vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 1 mL Volume, 100/pkg (p/n: 186000385C)
Mobile phase A:	0.1% (v/v) trifluoroacetic acid (TFA)
Mobile phase B:	0.1% (v/v) trifluoroacetic acid (TFA)
Data management:	Empower 3 Software

Gradient conditions for IL-12 (Figure 1)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.00	0.3	61	39	-
0.50	0.3	61	39	6
3.00	0.3	42	58	6
4.00	0.3	10	90	6
4.01	0.3	61	39	11
15.00	0.0	61	39	11

Gradient conditions for IL-12 at higher flow rate (Figure 2)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.000	1.8	65	35	-
0.083	1.8	65	35	6
0.500	1.8	45	55	6
0.650	1.8	10	90	6
0.660	1.8	65	35	11
5.000	0.0	65	35	11

Gradient conditions for EPO (Figure 4)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.00	0.3	63	37	-
0.50	0.3	63	37	6
3.00	0.3	40	60	6
4.00	0.3	10	90	6
4.01	0.3	63	37	11
15.00	0.0	63	37	11

Gradient conditions for Abatacept (Figure 5)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.00	0.2	60.5	39.5	-
0.50	0.2	60.5	39.5	6
3.00	0.2	53.0	47.0	6
4.00	0.2	10.0	90.0	6
4.01	0.2	60.5	39.5	11
15.00	0.0	60.5	39.5	11

Gradient conditions for Rituximab (Figure 6)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.00	0.3	62	38	-
0.50	0.3	62	38	6
5.50	0.3	57	43	6
6.50	0.3	10	90	6
6.51	0.3	62	38	11
15.00	0.0	62	38	11

Results and Discussion

A protein drug substance or drug product is usually dissolved in a formulation buffer, for which histidine is commonly used.[3] In a formulation, rHSA is also often used as an excipient to increase the stability and to act as a carrier protein.[4] In this experiment, L-histidine and rHSA were added intentionally to the protein solutions to mimic a formulation buffer. In order to be quantified, proteins need to be separated chromatographically from both histidine and rHSA. On reversed-phase columns, histidine elutes in the void. So, the task was to separate the protein drug from rHSA, and construct a calibration curve for quantification that would be valid even without the presence of rHSA. The molecular mass of rHSA is about 67 KDa.

Interleukin-12 (IL-12)

Interleukin-12 (IL-12) is a key immunoregulatory cytokine with a molecular mass of 70 kDa. It has been suggested that IL-12 plays a vital role in treating diseases such as viral and bacterial infections and cancers.[5]

Figure 1A shows IL-12 and rHSA separated on a BioResolve RP mAb Polyphenyl Column in under 4 minutes, with a resolution of 3.5 between the column bound rHSA and IL-12. Figure 1B shows peaks from various concentrations of IL-12. Based on the peak area, a calibration curve was constructed (bottom panel of

Figure 1C). Two linear dynamic ranges were identified from 0.01 to 0.1 µg and from 0.1 to 2 µg. The mass load (x) was calculated back from the peak area (y) and the linear equation obtained from the calibration curve, and it was then compared with the experimental mass load. The % error was calculated as (calculated mass load – experimental mass load)/experimental mass load *100%. A residual plot was constructed showing % error vs. mass load (top panel of figure 1c). For both linear ranges, the % error was less than 8% for all mass loads.

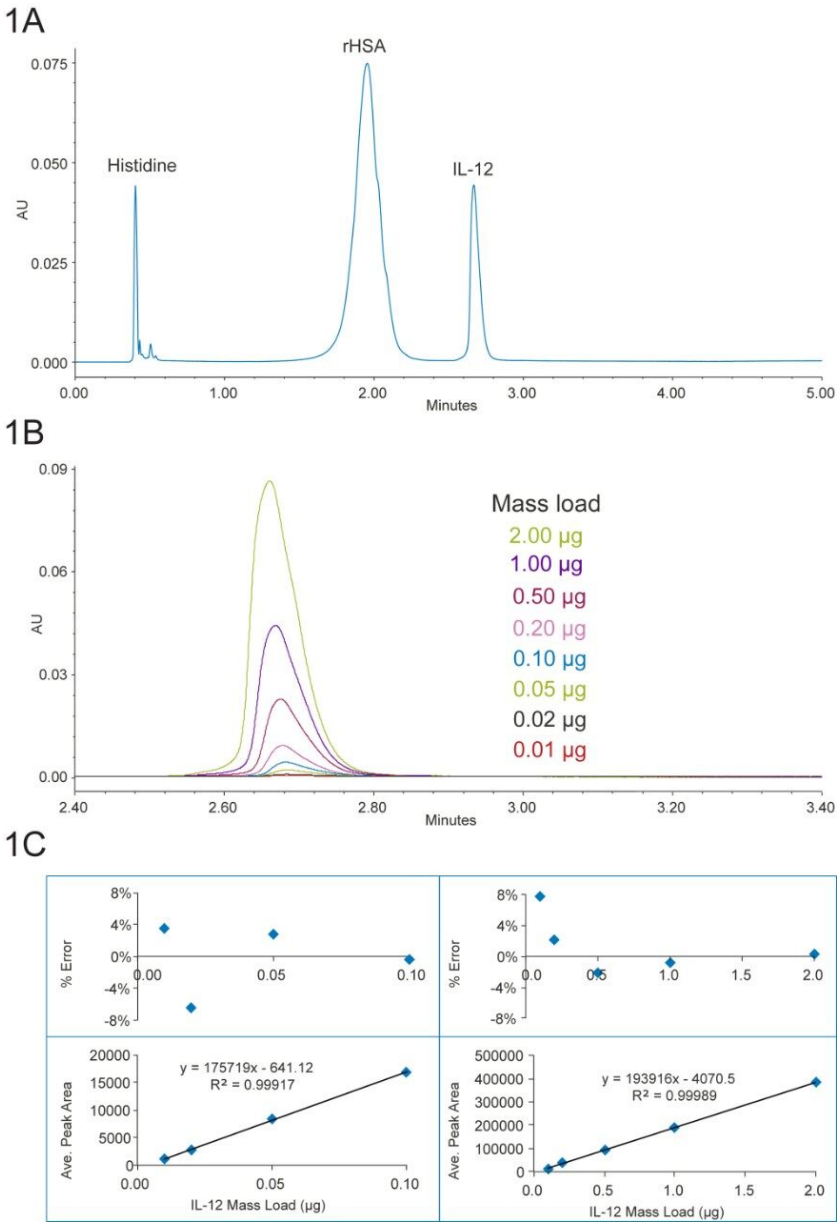

Figure 1. (A) One µg of IL-12 as separated from histidine and rHSA on a BioResolve RP mAb Polyphenyl Column. The flow rate was 0.3 mL/min. (B) Chromatogram overlays at various injected masses of IL-12 as separated from rHSA on the BioResolve RP mAb Polyphenyl Column. The flow rate was 0.3 mL/min. (C) IL-12 peak area calibration data (0.01–2 µg) were constructed from Figigure 1B information. Two linear ranges (0.01–0.1 µg and 0.1–2 µg) were identified that resulted in a calculated mass load error of less than 8%. The flow rate was 0.3 mL/min.

Since the BioResolve RP mAb Polyphenyl Column is packed with solid-core particles, higher flow rate and faster separation can be employed without sacrificing chromatographic performance, due to the excellent mass transfer (kinetic) property of these particles. As seen in Figure 2A, IL-12 and rHSA was separated under 1 minute at a flow rate of 1.8 mL/min. The resolution is the same as when the sample was separated at 0.3 mL/min with the same gradient slope (data not shown). Figure 2B shows the calibration curve and residual plot with the flow rate of 1.8 mL/min. The mass load % error was within 10% for the two linear dynamic ranges (0.1–0.5 µg and 0.5–5 µg). These results demonstrate that BioResolve RP mAb Polyphenyl Columns can be used for high throughput protein quantification in formulation buffer.

Injection-to-injection sample carryover from the column can be a problem for many reversed-phase protein separations, making quantification inaccurate. The BioResolve RP mAb Polyphenyl Column has been designed to have very low carryover by way of high coverage of the specially designed polyphenyl bonding. A double gradient was used to determine the carryover from the column. This example is shown in Figure 3A.

The sample was injected and the components were separated during the first gradient. After completion of the first gradient, a second gradient was started immediately without injecting the sample. If the column were to show carryover, IL-12 would be seen to elute at the indicated retention time. The ratio of the IL-12 peak areas between the second gradient and the first gradient is calculated as the percent carryover from the column. In Figure 3A, the top panel shows the chromatogram, the bottom panel shows the pressure trace, and arrows indicate where IL-12 is supposed to elute from the carryover determining gradient run. Figure 3B is a zoomed view of the y-axis. A small peak can be seen at a retention time of 10.6 minutes, which corresponds to IL-12 column carryover. The IL-12 carryover from the column is calculated as 0.11%, which limits the linear dynamic range to approximately 2–3 orders of magnitude. Additional blanks would be added to extend the linear dynamic range of the assay.

There is a possibility that the carryover could be masked by the carrier protein rHSA, with it potentially blocking the non-specific binding sites throughout the column. However, similar carryover was observed for a neat injection of IL-12 (0.08%).

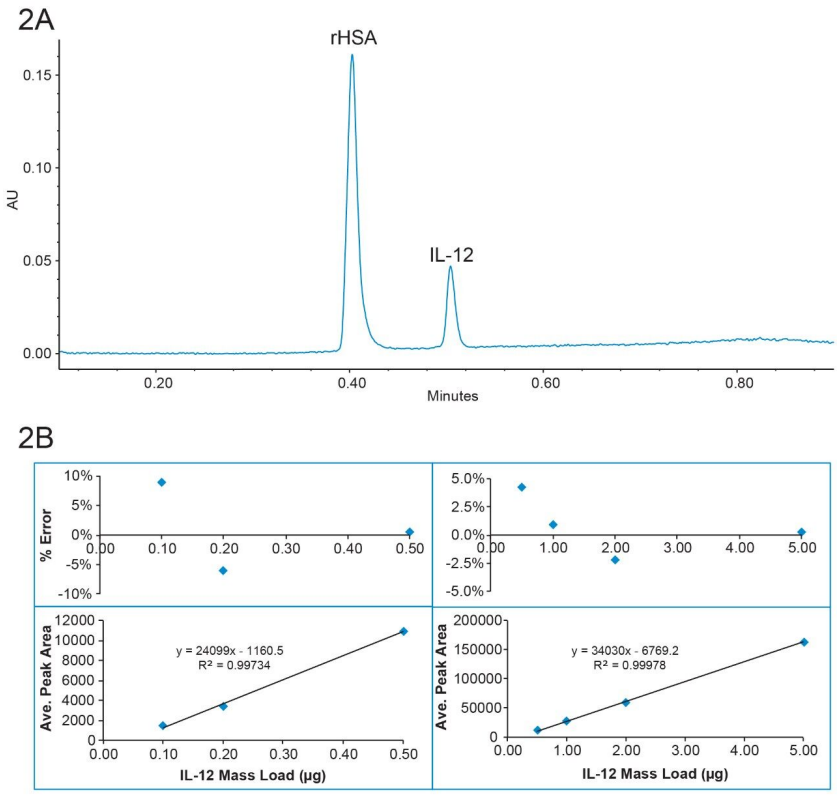

Figure 2 (A) One µg of IL-12 as separated from rHSA on a BioResolve RP mAb Polyphenyl Column. The flow rate was 1.8 mL/min. (B) IL-12 peak area calibration data (0.1–5 µg) were constructed from Figure 2A information. Two linear ranges (0.1–0.5 µg and 0.5–5 µg) were identified that resulted in calculated mass load error of less than 10%. The flow rate was 1.8 mL/min.

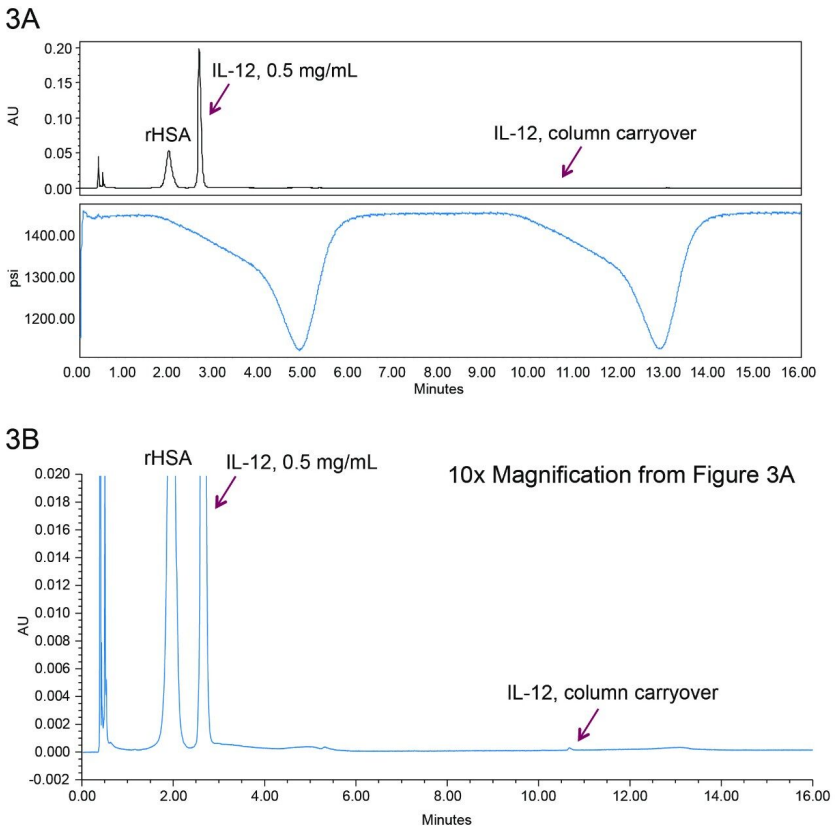

Figure 3. (A) Injection-to-injection protein carryover. (B) Small IL-12 carryover peak was observed in the carryover determining gradient run.

Erythropoietin (EPO)

EPO is a biotherapeutics glycoprotein that stimulates a patient's production of red blood cells. It is used to treat anemia resulting from kidney failure or cancer treatment.[6] The molecular mass of EPO is approximately 30.4 KDa.

Figure 4 shows various concentrations of EPO separated from rHSA on a BioResolve RP mAb Polyphenyl Column, and the insert shows the corresponding calibration curve and the residual plot. EPO was well resolved from rHSA with a resolution of >5. The linear dynamic range is from 0.5 to 10 µg with an R^2 value of 0.9999. The mass load % error is within 3%. Column carryover for EPO was not detectable.

Abatacept (Marketed as Orencia)

Abatacept is a Fc-fusion protein used to treat autoimmune diseases such as rheumatoid arthritis.[7] The molecular mass of Abatacept is about 90 KDa.

Figure 5 shows various concentrations of abatacept separated from rHSA. Although the separation was not baseline resolved, a linear dynamic range from 0.2 to 5 µg was achieved with R^2 = 0.9999. The mass load % error is within 3%. Column carryover for abatacept was undetectable.

Rituximab

Rituximab is a monoclonal antibody that treats certain autoimmune diseases and types of cancer.[8] The molecular mass of rituximab is around 150 kDa.

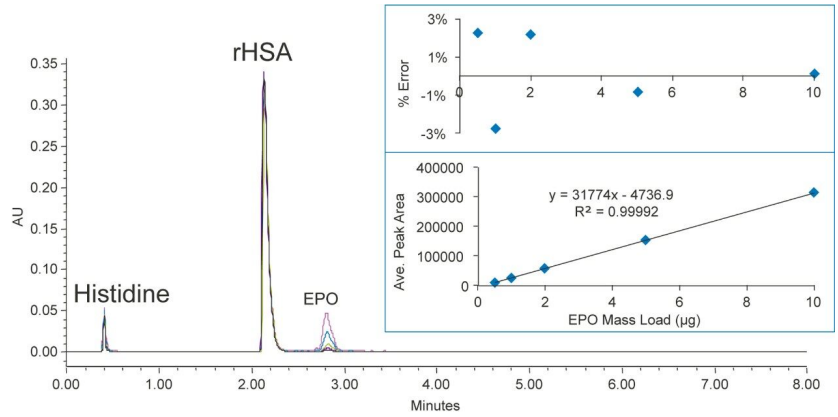

Figure 4. EPO chromatograms with peak area calibration curve (0.5–10 µg) and residual plot.

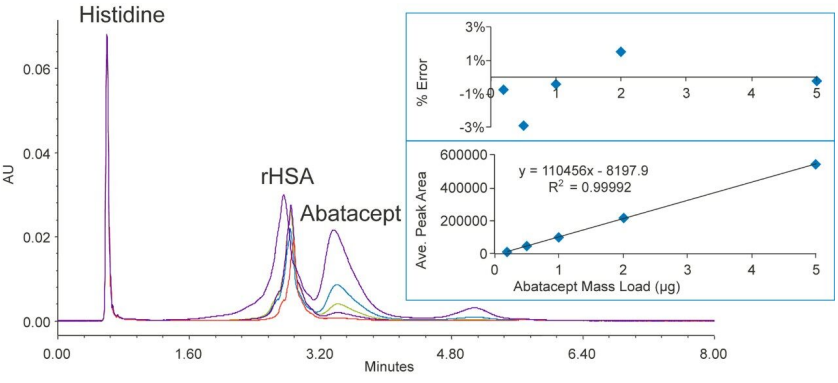

Figure 5. Abatacept chromatograms with peak area calibration curve (0.2–5 µg) and residual plot.

Figures 6 shows the chromatograms of various concentrations of rituximab separated from rHSA. For these separations, the column temperature was increased from 60 to 80 °C to ensure full recovery of the injected

rituximab. Each separation took less than 10 minutes. From these data, a calibration curve was constructed, and the linear dynamic range was found to be from 0.1 to 5 µg with an R2 value of 0.9993. The residual plot shows that the calculated mass load % error is <12%. Column carryover for rituximab was not detectable.

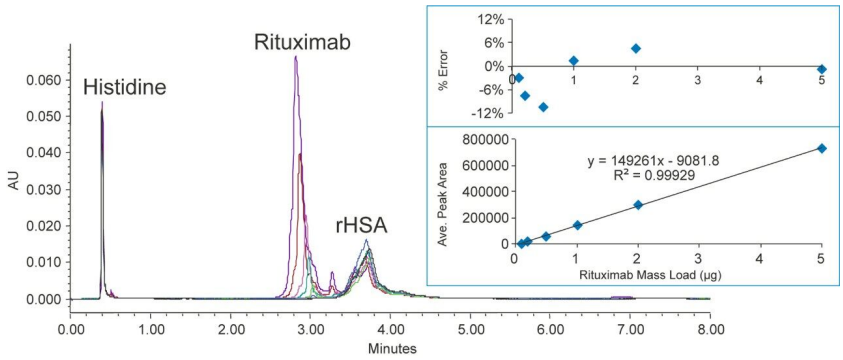

Figure 6. Rituximab chromatograms with peak calibration curve (0.1–5 µg) and residual plot, 80 °C.

Conclusion

The quantification of proteins as they exist in various biotherapeutic drug formulations is important in drug substance and drug product testing. In some cases, a formulation buffer might prohibit the use of simple UV absorbance measurements. There may even be cases, such as when using Immobilized Metal Affinity Chromatography (IMAC) with imidazole buffers, where purification intermediates cannot be quantified with direct UV absorbance measurements. In these cases, a chromatographic separation based on reversed-phase or affinity chromatography is needed to separate and accurately quantitate the drug from interfering UV absorbing contaminants.

In this application note, we have shown that the BioResolve RP mAb Polyphenyl Column, when paired with simple 0.1% TFA water/acetonitrile mobile phases, works well as a tool to use in the quantification of various types of proteins that might be formulated in buffers containing L-histidine and rHSA. With the methods presented here, it was possible to obtain excellent linearity, low % error, good dynamic ranges, along with extremely low column injection-to-injection sample carryover to allow for the routine, high throughput analyses.

References

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8. https://en.wikipedia.org/wiki/Rituximab

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720006233, March 2018