Poster Note 64689

Characterization of a Biologic Therapeutic: Reversed Phase Analysis of Protein and Excipients

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Overview

Purpose: To develop a method for the simultaneous separation of therapeutic proteins and amino acid excipients.

Methods: A 2D approach for the separation of protein therapeutics and underivatized amino acid excipients. An integrated UHPLC system with a UV and universal charged aerosol detection offering multi-mode detection for the simultaneous analysis of both non-chromophore and chromophore compounds was employed.

Results: A method for the determination of label free amino acids and proteins from a commercial therapeutic protein formulation using multi-modal UV and charged aerosol detection is described. Multi-modal UV and charged aerosol detection in an integrated system provides a suitable means for the analysis of analytes consisting of both chromophore and non-chromophore species. The detectors are orthogonal and complimentary in nature so that more compounds in the sample can be detected.

Introduction

Therapeutic proteins (antibodies and vaccines) vary considerably due to the nature and dose of the protein molecule. Vaccine formulations differ from therapeutic antibody formulations since they often include an additional component or adjuvants for immune-enhancement. Often surfactants such as Polysorbates are ubiquitous to these protein formulations because of their effectiveness in protecting many proteins.

Unwanted aggregation is a major degradation pathway of protein therapeutics during their storage [1]. The protein structure is susceptible to aggregation-prone phase transitions which are dependent on pH, temperature, and protein concentration. Stabilization of protein formulations can be enhanced through the addition of specific amino acids excipients as well as other compounds such as surfactants and sugars [2, 3]. Of all the possible amino acids only a selected few are commonly used as excipients in protein therapeutic formulations. These include Arginine (Arg), Aspartic acid (Asp), Glutamic acid (Glu), Lysine (Lys), Proline (Pro), Glycine (Gly), Histidine (His), and Methionine (Met) [4]. Amino acids such as Lys and Arg are positively charged, while Glu and Asp are negatively charged amino acids. The amino acids present in protein formulations serve as buffers, bulking agents, stabilizers, and antioxidants [5]. For example, glutamic acid and histidine can help adjust the final pH and replace organic buffers such as acetate and citrate, respectively. Methionine can be included as an antioxidant in formulations and arginine has been shown to be highly effective at suppressing aggregation in both liquid and lyophilized formulations while glycine, proline, serine, and alanine can partially serve in this capacity as well [6].

The UHPLC analytical system includes a sensitive diode array detector (DAD) for measurement of compounds with suitable chromophores. The Charged Aerosol Detector is a sensitive universal detector that is used to measure compounds that lack a chromophore. Charged Aerosol detection (CAD) is a mass sensitive technique for determining levels of any non-volatile and many semi-volatile analytes after separation by liquid chromatography. This technique provides consistent analyte response independent of chemical characteristics. An analyte's response does not depend on optical properties, like with UV-vis absorbance, or the ability to ionize, as with mass spectrometry. The presence of chromophoric groups, radiolabels, ionizable moieties, or chemical derivatization is not needed for detection. Thus non-chromophore drug impurities can be easily monitored by CAD.

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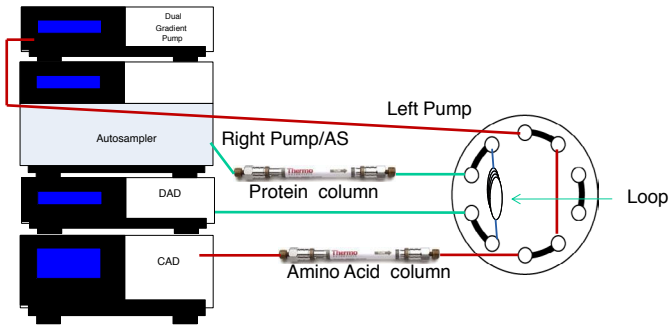
FIGURE 1. 2D Separation of Therapeutic Protein and Amino Acid Excipients



Methods

The Thermo Scientific™ UltiMate™ 3000 UHPLC 2D Liquid Chromatography system consisted of a DGP-3600RS pump, a WPS-3000TRS autosampler, a TCC-3000RS column oven with 10-port column switching valve, a DAD-3000(RS) and a Veo RS Charged Aerosol Detector.

All amino acid standards are prepared using 0.1N HCl as the diluent. A mixture of amino acid standards with each compound at a concentration of 100 µg/mL was prepared in water. Another vial was prepared with the Amino acid Standard H from Pierce by diluting the standard 1:10 in water.

A freeze dried powder of a commercial protein therapeutic containing a mixture of surfactant, amino acids, sugars and MAb protein was purchased and weighed and finally diluted with water to a final concentration of 2.0 mg/mL.

Dimension 1: Method for Analysis of Therapeutic Protein

HPLC column:	Thermo Scientific™ MAbPac RP, 2.1 x 50 mm
Mobile Phase A:	0.1% Trifluoroacetic acid (TFA) in Water
Mobile Phase B:	0.1% TFA in Acetonitrile
Mobile Phase C:	0.1% Formic acid in 90% Water and 10% Acetonitrile
Column Temp.:	75 °C
Detector:	DAD, 20 Hz data rate, 0.5 s response time
Flow Rate:	0.250 - 0.3 mL/min
Gradient:	

No	Time	Flow [ml/min]	%B	%C	Curve
1	-5.000	Equilibration			
2	-5.000	0.250	0.0	100.0	5
3	*New Row*				
4	0.000	Run			
5	0.000	0.250	0.0	100.0	5
6	1.000	0.250	0.0	100.0	5
7	1.100	0.300	25.0	0.0	5
8	12.000	0.300	50.0	0.0	5
9	22.000	0.300	50.0	0.0	5
10	24.500	0.300	25.0	0.0	5
11	25.000	0.300	0.0	100.0	5
12	*New Row*				
13	25.000	Stop Run			

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Dimension 2: Method for Analysis of Underivatized Amino Acid Excipients

HPLC column:	Thermo Scientific™ Acclaim™ PA2, 2.2 µm, 2.1 x 250 mm
Mobile Phase A:	0.15% Nonafluoropentanoic acid (NFPA) in H2O
Mobile Phase B:	0.2% TFA in Acetonitrile
Mobile Phase C:	5% Acetic acid
Column Temp.:	Ambient
Detector:	CAD, 20 Hz data rate, 5 s response time, 50 °C evaporation temp., 1.00 PFV
Flow Rate:	0.4 - 0.5 mL/min
Gradient:	

No	Time	Flow [ml/min]	%B	%C	Curve
1	-5.000	Equilibration			
2	-5.000	0.400	2.0	8.0	5
3	*New Row*				
4	0.000	Run			
5	0.000	0.400	2.0	8.0	5
6	1.000	0.400	2.0	8.0	5
7	2.500	0.500	3.0	5.0	5
8	10.000	0.500	8.0	3.0	5
9	14.000	0.500	18.0	2.0	5
10	19.000	0.500	30.0	2.0	5
11	24.000	0.500	30.0	2.0	5
12	25.000	0.500	3.0	8.0	5
13	*New Row*				
14	25.000	Stop Run			

Data Analysis
Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, 7.2

Results and Discussion

The chromatographic separation of therapeutic protein and amino acid excipients was performed using a 2D approach as illustrated in Figure 1. The protein was separated as shown in Figure 2 using a MAbPac RP column with a water: acetonitrile gradient with each solvent containing TFA as an ion pairing agent. A heart cut from 0.5 to 0.8 minutes containing the polar amino acids present in the sample was transferred to a second reversed phase column via a switching valve. The separation of underivatized amino acid excipients and other compounds such as sucrose was then accomplished within 25 minutes using a long RP column. The main perfluorinated carboxylic acid used was NFPA. It has both volatile and strong ion pairing characteristics and was used in addition to TFA. The gradient used for the separation of amino acids was optimized by manipulating the levels of ion-pairing reagents, pH and level of organic solvents. Both TFA and NFPA are acidic and there was concern that the low pH would adversely affect column performance over time. By including acetic acid during the gradient, the resulting elevation in pH enabled the routine use of the column without deterioration in performance during the development of this method. An additional advantage of this approach was to adjust the pH during elution thereby controlling the bulk charge of amino acids and the separation of closely eluting peaks.

FIGURE 2. Dimension 1. Separation of Protein Therapeutic using Reversed Phase Chromatography with Ion-pairing.

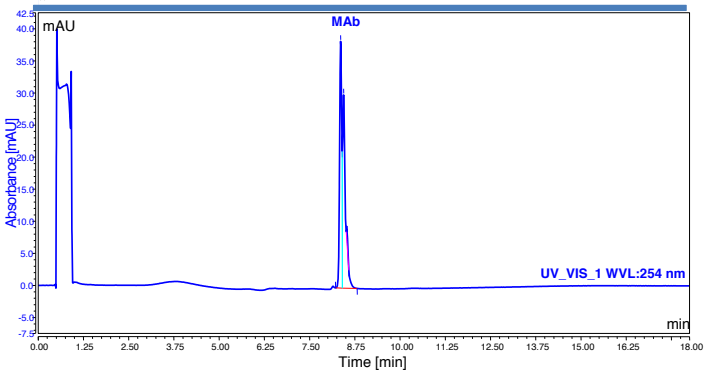
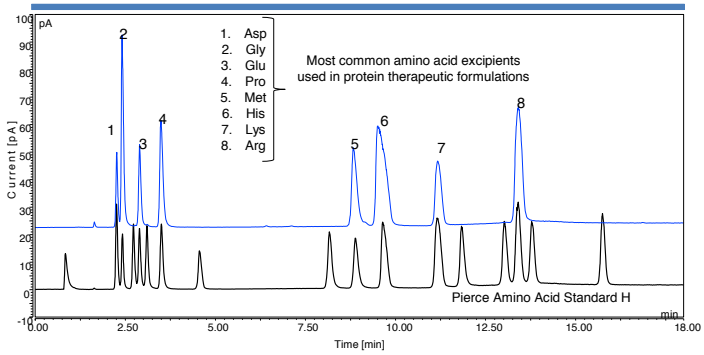


FIGURE 3. Dimension 2: Analysis of Underivatized Amino Acid Excipients (0.4 µg on column).



The Acclaim PA2 column used for the separation for amino acids as described in this method provided good data precision since the %RSD ranged from 0.54 to 3.87 percent as shown in Table 1 below. The transfer of an aqueous sample via column switching from the MAbPac RP column to the Acclaim PA2 column did not appear to elevate the %RSD for early eluting amino acids. The highest %RSD value shown in Table 1 was obtained for methionine and this may be attributed to its relative instability compared to other amino acids [5].

TABLE 1. Precision data metrics for analysis of underivatized amino acids

Amino Acid	Asp	Gly	Glu	Pro	Met	His	Lys	Arg
Direct inject %RSD	1.41%	0.92%	1.02%	1.03%	2.63%	2.44%	1.60%	1.45%
Heart Cut %RSD	1.93%	0.54%	1.18%	2.58%	3.87%	1.63%	1.81%	1.81%

Calibration curves for MAb, sugar and amino acid excipients are shown in Figure 4. Sinc the charged aerosol detector produces a non-linear detector response a polynomial curv fit is used for sucrose and histidine calibration curves. The coefficients of determination (R2) values for the various components are shown in Table 2 and ranged from 0.9976 to 0.9998. The goodness of fit was greater than 0.997 for the compounds evaluated.

FIGURE 4. Calibration Curves for MAb, Sugar and Amino Acid Excipients

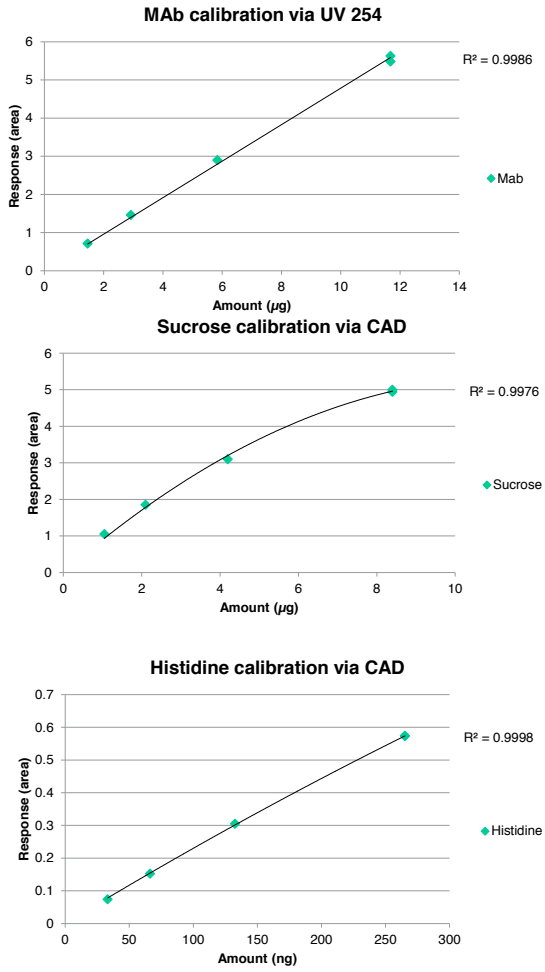


Table 2. Metrics for coefficient of determination (R^2) for major components of a commercial protein therapeutic.

Component	Detector	Regression	R² Values
MAb	UV	Linear	0.9976
Sucrose	CAD	Polynomial	0.9986
Histidine	CAD	Polynomial	0.9998
Histidine	CAD	Linear	0.9984

A commercial therapeutic protein formulation containing a mixture of surfactant, amino acids, sugars and protein was prepared in water. This solution was analyzed to demonstrate the simultaneous separation of these compounds using the 2D approach described. The diode array detector was able to detect the protein as illustrated in Figure 5 but the Polysorbate 80 surfactant was invisible since it is devoid of a suitable chromophore. The Charged Aerosol Detector is a sensitive universal detector designed for UHPLC and enables a wide dynamic range.

The wide dynamic range available with this detector is clearly illustrated since high levels of sucrose are shown along with lower levels of the amino acid histidine. The current method using 2D separation of proteins and excipients allowed for concentrations of the main formulation components to be measured (see Table 3).

FIGURE 5. Commercial Protein Formulation with Amino Acid Excipients (4 µL injected).

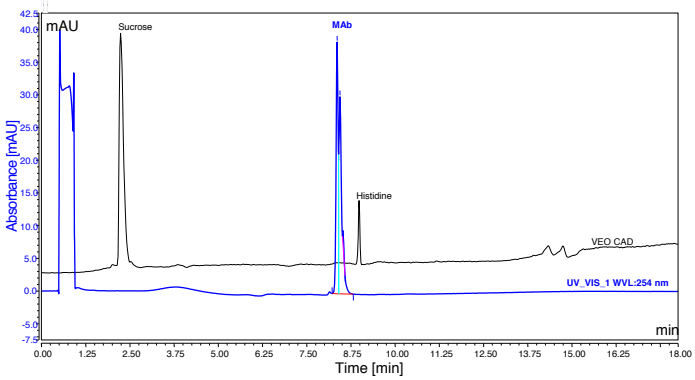


TABLE 3. Calculated concentrations of MAb, sugar and amino acid excipient present in a commercial protein therapeutic formulation.

Component	Found (mg/mL)	Expected (mg/mL)	% Recovery
MAb	4.42	4.58	96.5
Sucrose	3.14	3.3	95.1
Histidine	0.1	0.104	96.1

Conclusions

- The simultaneous separation and detection of protein therapeutics and amino acid excipient was demonstrated using 2D chromatography.
- A commercial protein formulation containing a mixture of surfactant, amino acids, sugars and protein was analyzed to demonstrate the successful capability of the method. The power of this automated approach offers the possibility to measure impurities. Use in a QA/QC environment would save time, money, and the need for extensive sample preparation.
- The method developed using column switching with both UV and charged aerosol detector demonstrated good precision (%RSD range 0.54 to 3.87) and high coefficient of determination (R^2) metrics (0.9976 – 0.9998) for components from a commercial protein therapeutic.

References

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