Poster Note 71849

2D Analysis of Protein Therapeutics and Amino Acid Excipients with Combined UV and Charged Aerosol Detection

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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 HILIC method for the determination of label free amino acids and proteins 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 drugs 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.

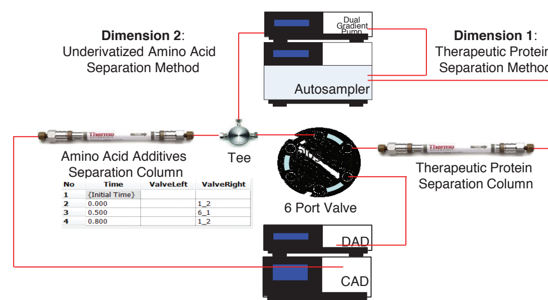
Introduction

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. 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. Of all the possible amino acids only a selected few are commonly used as excipients in protein therapeutic formulations. These include Arginine, Aspartic acid, Glutamic acid, Lysine, Proline, Glycine, Histidine, and Methionine. 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. 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.

The system provides sensitive DAD for those compounds with suitable chromophores. The Charged Aerosol Detector is a sensitive universal detector designed for UHPLC and provides a wide dynamic range for those 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 and gives greater sensitivity over a wider dynamic range. An analytes response does not depend on optical properties, like with UV-vis absorbance, or the ability to ionize, as with mass spectrometry (MS). 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.

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



No	Time	ValveLeft	ValveRight
1	{Initial Time}		
2	0.000		1_2
3	0.500		6_1
4	0.800		1_2

Methods

2D Liquid Chromatography using the Thermo Scientific™ UltiMate™ 3000 UHPLC system including: the UltiMate 3000 system consisting of a DGP-3600RS pump, WPS-3000TRS autosampler, TCC-3000RS column oven with 6-port column switching valve, DAD-3000(RS) and Veo RS Charged Aerosol Detector

Dimension 1: Method for Analysis of Therapeutic Protein

HPLC column:	Thermo Scientific™ Accucore™ 150 C4, 2.6 μm, 3.0 x 50 mm
Mobile Phase A:	0.1% TFA in Water
Mobile Phase B:	0.1% TFA in Acetonitrile
Mobile Phase C:	0.05% formic acid in 50% Water and 50% Acetonitrile
Column Temp.:	45 °C
Detector:	DAD, 10 Hz data rate, 0.5 s response time
Flow Rate:	0.350 - 0.4 mL/min
Gradient:	

No	Time	Flow [ml/min]	%B	%C	Curve
1	-4.000	Equilibration			
2	-4.000	0.350	0.0	100.0	5
3	*New Row*				
4	0.000	Run			
5	0.000	0.350	0.0	100.0	5
6	0.800	0.350	0.0	100.0	5
7	0.801	0.400	10.0	0.0	5
8	12.000	0.400	90.0	0.0	5
9	18.000	0.400	90.0	0.0	5
10	20.000	0.350	0.0	100.0	5
11	*New Row*				
12	20.000	Stop Run			

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Dimension 2: Method for Analysis of Underivatized Amino Acid Excipients

HPLC column:	Thermo Scientific™ Acclaim™ Trinity P1, 3.0 µm, 3.0 x 50 mm and Acclaim Trinity P1, 3.0 µm, 3.0 x 100 mm in series
Mobile Phase A:	Water, 0.05% formic acid
Mobile Phase B:	Acetonitrile
Mobile Phase C:	120 mM Ammonium Formate, pH 3.3
Column Temp.:	45 °C
Detector:	CAD, 20 Hz data rate, 5 s response time, 50 °C evaporation temp., 1.00 PFV
Flow Rate:	
Gradient:	

No	Time	Flow [ml/min]	%B	%C	Curve
1	-4.000	Equilibration			
2	-4.000	0.700	95.0	4.5	5
3	New Row				
4	0.000	Run			
5	0.000	0.700	95.0	4.5	5
6	0.800	0.700	94.0	4.5	5
7	0.801	1.000	94.0	4.5	5
8	5.500	1.000	68.0	7.0	5
9	9.500	1.000	40.0	32.0	5
10	18.500	1.000	40.0	38.0	5
11	19.000	1.000	95.0	4.5	5
12	20.000	1.000	95.0	4.5	5
13	New Row				
14	20.000	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 usin an Accucore 150 C4 column as shown in Figure 2 using 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 from the sample injection was transferred to a second column via a switching valve. The separation of underivatized amino acid excipients and several ions was then accomplished within 20 minutes using HILIC mode on the Acclaim Trinity P1 column as illustrated in Figure 3. The mixed mode Acclaim Trinity P1 column provides cation, anion and reversed phase separation characteristics. The gradient chose for the separation of amino acids and selected ions was adjusted by selecting appropriate ionic buffer strength, pH and level of organic solvents.

The mixed mode Acclaim Trinity P1 column packing material contains cation, anion and reversed phase separation characteristics. The gradient chosen for the separation of amino acids and selected ions was adjusted by selecting appropriate ionic buffer strength, pH and level of organic solvents.

FIGURE 2. Dimension 1: Analysis of Therapeutic Protein (8 µg on column).

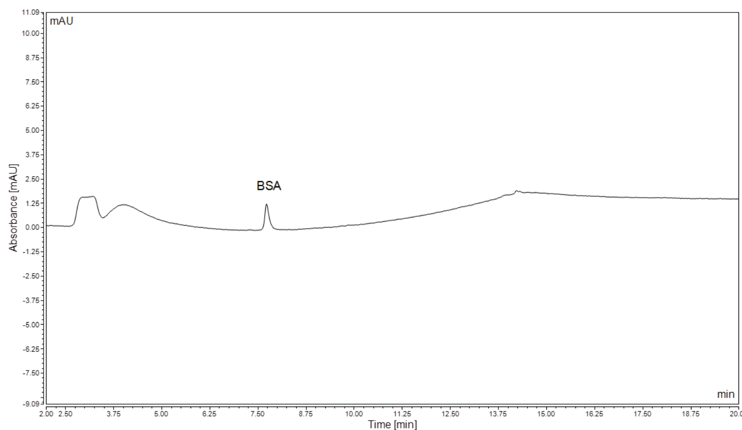
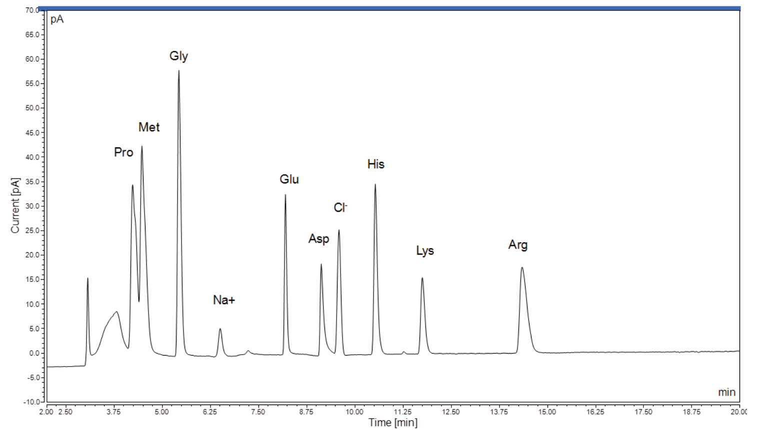


FIGURE 3. Dimension 2: Analysis of Underivatized Amino Acid Excipients (0.4 µg on column).



The Acclaim Trinity P1 column used for the separation for amino acids as described in this method, provided good data precision since the RSD ranged from 0.45 to 2.91 percent as shown in Table 1 below. In order to prevent peak shape issues related to the transfer of polar material from an aqueous sample onto the second column operating with high levels of organic solvents in HILIC mode, a bridge solvent was used. Initially the TFA mixture was flushed from the first column and the sample was injected with a mobile phase comprised of 0.05% formic acid in 50% Water and 50% Acetonitrile. This provided a suitable environment for the transfer of polar compounds to the second column. The transfer of an aqueous sample via column switching from the Accucore C4 column to the Trinity P1 column did elevate the %RSD for early eluting amino acids, proline and methionine. The remainder of the amino acids showed excellent reproducibility with %RSD below 1 percent..

TABLE 1. Precision data metrics for analysis of underivatized amino acids (1 µg on column n=6)

	Pro	Met	Gly	Glu	Asp	His	Lys	Arg
Avg Area	9.5714	14.7931	12.676	4.5296	4.2661	7.3595	4.3933	8.8612
%RSD	2.91%	2.80%	0.84%	0.56%	0.65%	0.45%	0.78%	0.71%

The calibration curves for amino acids shown in Figure 4 use an inverse axis in order to provide a better fit or the non-linear detector response observed with the charged aerosol detector. The coefficient of determination (R^2) for the calibration curve ranging from 0.1 – 1 ug on column are shown in Table 1. The goodness of fit was greater than 0.985 for all compounds evaluated.

FIGURE 4. Calibration Curves for Amino Acid Excipients

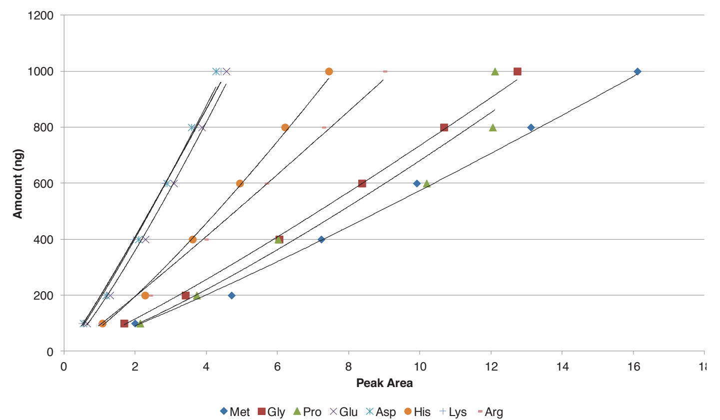


TABLE 2. Coefficient of determination (R^2) metrics for analysis of underivatized amino acids by CAD from 100 – 1000 ng on column.

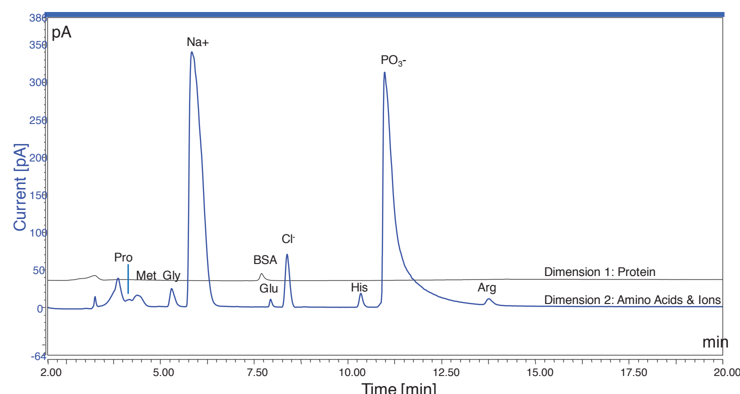
	Pro	Met	Gly	Glu	Asp	His	Lys	Arg
Coefficient of determination (R^2)	0.985	0.987	0.998	0.996	0.99	0.993	0.993	0.989

A mixture of surfactant, amino acids, ions and protein which represents a mock protein formulation with amino acid excipients was prepared as shown in Table 3. This solution was injected to demonstrate proof of concept for the simultaneous separation of these compounds using the 2D approach described. The protein and surfactant were separated on the Accucore 150 C4 column. 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 structure. The ions and amino acids transferred and separated on the Acclaim Trinity P1 column were detected using the charged aerosol detector as shown in Figure 5. The Charged Aerosol Detector is a sensitive universal detector designed for UHPLC and provides a wide dynamic range. The wide dynamic range available with this detector is clearly illustrated since high levels of sodium and phosphate (100 µg on column) are shown along with lower levels of amino acids (200 ng on column).

TABLE 3. Composition of a mixture of surfactant, amino acids, ions and protein representing a mock protein formulation with amino acid excipients.

Ingredient	Concentration (µg/mL)
Sodium Chloride	200
L-Histidine	20
Polysorbate 80	1000
Sodium Phosphate	10000
Protein (BSA)	1000

FIGURE 5. Mock Protein Formulation with Amino Acid Excipients (10 µL injected).



Conclusions

- The simultaneous separation and detection of protein therapeutics and amino acid excipient was demonstrated using 2D chromatography.
- A mock protein formulation containing a mixture of surfactant, amino acids, ions and protein was injected to demonstrate the successful capability of the method.
- The method using the charged aerosol detector demonstrated good precision (%RSD range 0.45 – 2.91) and high coefficient of determination (R^2) metrics (0.985 – 0.998) for underivatized amino acids.

References

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