Determination of Proteins and Carbohydrates by 2D HPLC (RPLC and HILIC) with Charged Aerosol and Ultraviolet Detection

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Poster Note 71863

Overview

Purpose: Characterization of a biologic using an analytical method for the determination of carbohydrates and proteins in a sample by HPLC using Corona charged aerosol and ultraviolet detectors.

Methods: The method analyses an aqueous sample with injection on a reverse phase column and transfers the unretained carbohydrates to a HILIC column via sample loop and organic solvent addition.

Results: The method is quantitative for the protein and seven carbohydrates. Corona charged aerosol detection is over 5-fold more sensitive for the protein (BSA) than UV at 280 nm.

Introduction

Many of today's pharmaceutical formulations use proteins and other biotechnological APIs. Along with these newer formulations come different excipients, namely surfactants, amino acids, salts, and carbohydrates. Carbohydrates are used to assist in the stabilization of the proteins in solution, and are used in different amounts, depending on the protein and the product requirements. Many carbohydrates are found in these formulations, including the sugars glucose, sucrose, lactose, maltose, and trehalose and the sugar alcohols sorbitol and mannitol.

The quantitation of these carbohydrates in bioformulations can be complicated, since the carbohydrates are typically separated using HILIC where the protein API would irreversibly retain, but when using reverse phase, they have no retention.

A method was developed that separates the carbohydrate excipient from the protein, and quantifies both simultaneously. The method injects the sample on to the reverse phase column, where the protein is retained, and the carbohydrates are not. These are captured into a 500 μL sample loop while being mixed with acetonitrile in preparation for the HILIC analysis. Once the carbohydrates are captured, the gradient elution of the protein is conducted, as well as the analysis of the carbohydrates by HILIC simultaneously.

The method uses the traditional UV at 214 and 280 nm for the protein analysis, but for method development and comparison for sensitivity, a Thermo Scientific™ Dionex™ Corona™ Veo™ SD CAD was used. The carbohydrate method also used a Corona Veo SD, providing a sensitive and linear calibration of each of the seven carbohydrates.

Below, in Figure 1, is the method development system schematic, using a dual-gradient HPLC pump, the 10-port valve, and detectors. The CAD used for the protein analysis is not necessary in final system, which would simplify the system.

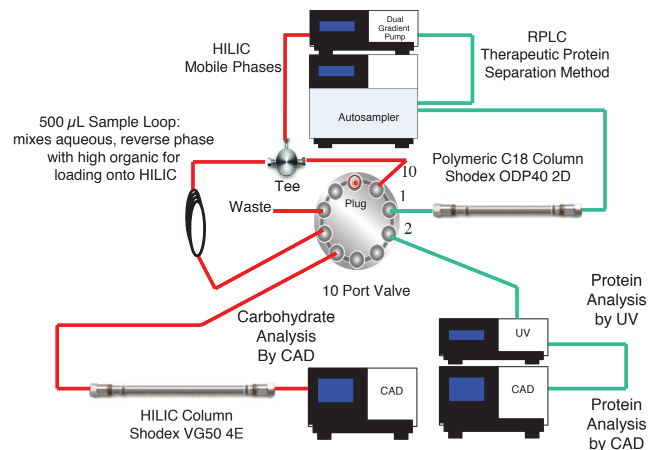

FIGURE 1. System schematic of 2-D HPLC method development system using a sample loop between the RPLC and HILIC gradient elution methods.

Time (min)	Flow Rate (m/min)	%A1	%B	%C
-5	0.25	80	0	20
0	0.25	80	0	20
2	0.25	80	0	20
10	0.25	20	20	60
16	0.25	20	20	60
16	0.25	80	0	20
18	0.25	80	0	20

Time (min)	Flow Rate (m/min)	%A2	%B	%C	Left Valve
-5.00	0.50	5	0	95	1_2
0.00	0.50	5	0	95	
0.90	0.50	5	0	95	10_1
1.35	0.50	5	0	95	1_2
1.35	0.50	12	15	73	
1.90	0.85	12	15	73	
11.00	0.85	12	33	55	
16.00	0.50	12	33	55	
18.00	0.50	5	0	95	

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Results

Calibration

The HILIC method is designed to provide resolution between the seven, different carbohydrates. There is partial overlap of the sorbitol, mannitol, and glucose analytes, but they are rarely found together in a single formulation. The other carbohydrates are well resolved.

Standards were prepared from 3.9 to 1000 µg/mL in water and injected in triplicate.

An overlay of chromatograms of the three, separate carbohydrate combinations, at 1000 µg/mL (2000 ng on column (ng o.c.)), is shown in Figure 2. Maltose and lactose are reducing sugars, which tend to react with the amines of most HILIC columns and thereby reducing response, but the Shodex VG-50 column mitigates this effect by using hindered amines on the solid phase. This provided better response for these carbohydrates. A plot of the calibration data for five of the carbohydrates, with linear regression fits, is shown in Figure 3. All of the linear correlation coefficients, R^2, were greater than 0.997, as shown in Table 1.

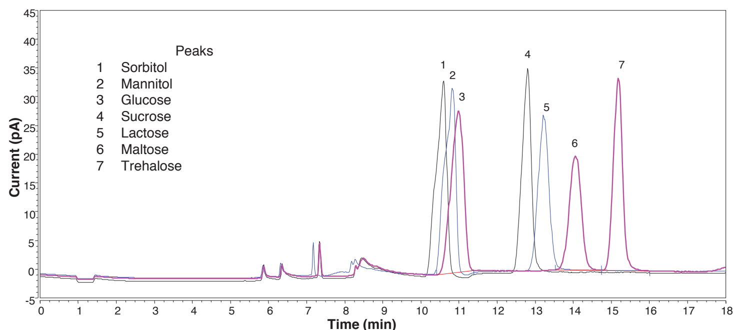

FIGURE 2. Seven carbohydrates by 2D-HPLC-CAD. All amounts of carbohydrates at 2000 ng o.c.

The precision of the triplicate injections was high, with peak area percent relative standard deviations between 0.3 and 4.4% with the higher values approaching the LOQ value for the analyte.

The determination of limits of detection (LOD) and quantitation (LOQ) were made using signal-to-noise ratio values of the peak with baseline noise measured near the peak. These values are also listed in Table 1. Generally, the LOD values for all of the carbohydrates were between 4–9 ng o.c., and the LOQ values were between 14–29 ng o.c. The expected values for these carbohydrates would be lower, but there is some peak widening due to the exchange of the carbohydrates from the reverse phase column to the HILIC method.

Also in Table 1 are the sensitivity and regression correlation values for the BSA, both by CAD and UV at 280 nm. The calibration curve for BSA by HPLC-CAD is shown in Figure 4, from amounts of 15.6 to 2000 ng o.c.

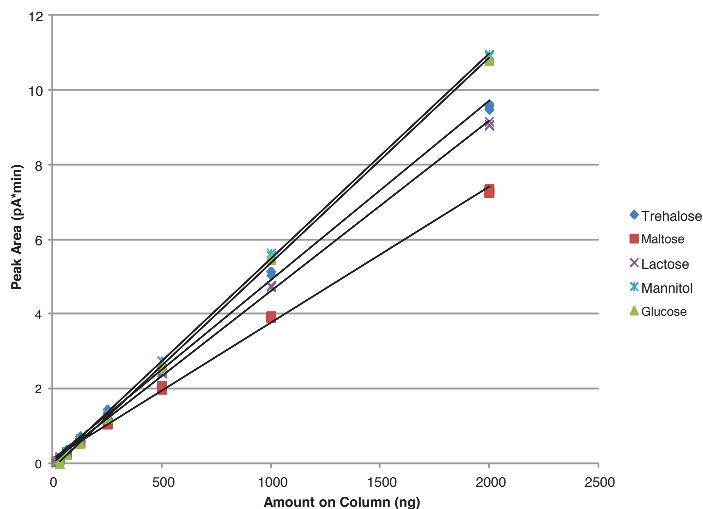

FIGURE 3. Linear calibration curves for four sugars and a sugar alcohol by HPLC-CAD, 15.6 – 2000 ng o.c

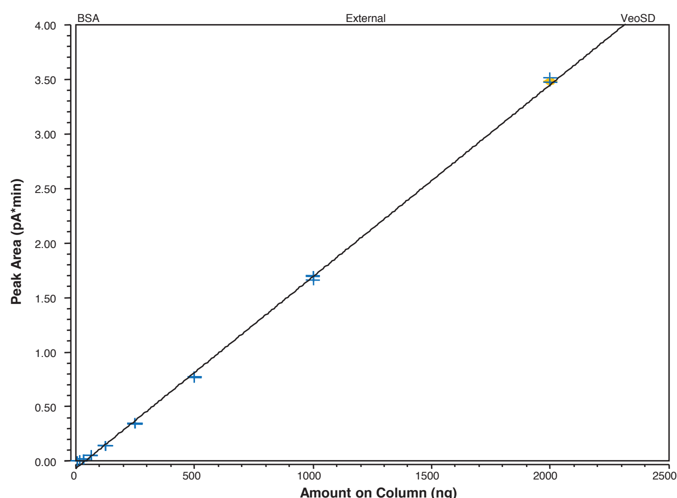

FIGURE 4. Linear calibration curves for BSA protein by HPLC-CAD, 15.6 – 2000 ng o.c

Table 1. Experimental sensitivity and linear calibration regression coefficients (R^2) for seven carbohydrates, and BSA, by CAD and UV at 280nm.

Analyte	LOD (ng o.c.)	LOQ (ng o.c.)	Linear R^2
Sorbitol	5.9	20	0.9984
Mannitol	5.6	19	0.9998
Glucose	8.6	29	0.9997
Sucrose	4.3	14	0.9978
Lactose	5.3	18	0.9993
Maltose	6.8	22	0.9982
Trehalose	4.5	15	0.9985
BSA-CAD	3.5	12	0.9993
BSA-UV280	24	79	0.9991

BSA Quantitation

The surrogate protein, BSA, was used in all of the samples to investigate the stability of the analysis, and to provide a means of comparing the response characteristics of UV at two wavelengths and the Corona Veo detector.

The UV wavelengths, 214 and 280 nm, were chosen as they are frequently used to determine protein in samples. With the mobile phases used, the protein responded well at 214 nm, but due to baseline issues, the peak could not be integrated accurately.

A comparison of 125 ng o.c. of BSA, by CAD and UV at 280 nm is shown in Figure 5. Both peaks have similar areas, but the quality of the baseline on CAD resulted in an LOQ value of 12 ng o.c., whereas on the UV at 280 nm, the LOQ value was determined at 79 ng o.c.

Recovery

The 1000 ng o.c. solutions were injected in duplicate and analyzed as samples to determine accuracy of the method and calibration. The summary of results is shown in Table 2. The range of recovery values for the seven carbohydrates was from 97.5 to 101.3 %. For the BSA, accuracy was 96% by CAD and 101.9 % by UV at 280 nm.

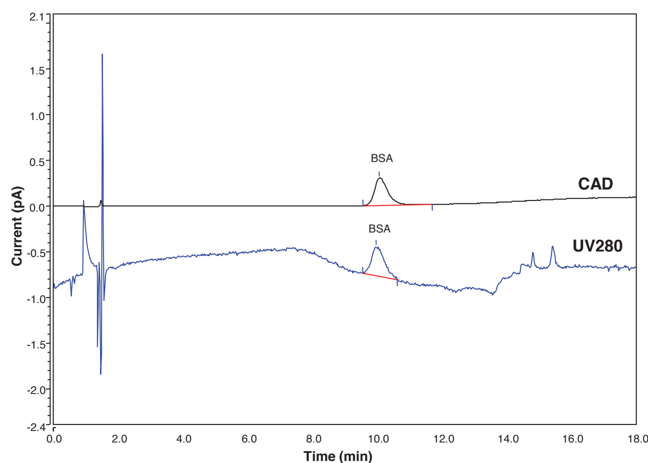

FIGURE 5. Overlaid HPLC chromatograms of BSA at 125 ng o.c. by UV at 280 nm (blue) and CAD (black).

Table 2. Experimental recovery data for seven carbohydrates, and BSA, by CAD and UV at 280nm.

Analyte	Amount (ng o.c.)	Found (ng o.c.)	Recovery (%)
Sorbitol	1000	975.0	97.5
Mannitol	1000	1013	101.3
Glucose	1000	1008	100.8
Sucrose	1000	988.4	98.8
Lactose	1000	990.6	99.1
Maltose	1000	1013	101.3
Trehalose	1000	1003	100.3
BSA-CAD	1000	960.4	96.0
BSA-UV280	1000	1019	101.9

Conclusions

- A simple workflow capable of characterizing a biologic formulation was developed that will analyze both a protein and a carbohydrate in a bioformulation, accurately and precisely using RPLC and HILIC, respectively
- CAD can be used to accurately determine carbohydrate and protein in bioformulations.
- CAD was found to be over five-fold more sensitive for protein (BSA) than UV at 280 nm, in the method development system. The second CAD is not necessary for the quantitation of the protein, for a simplified system.
- LOQ values for the carbohydrates ranged from 14–29 ng o.c.

Acknowledgements

We would like to thank Dr. Ron Benson of Showa Denko America for the use of the Shodex VG50 column used in this work.

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PO71863-EN 1115S

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