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

Marc Plante[1], Ian N Acworth[1], Bruce Bailey[1], Evert-Jan Sneekes[2], Frank Steiner[2]
[1]Thermo Fisher Scientific, Chelmsford, MA,
[2]Thermo Fisher Scientific, Dornierstrasse 4, Germering, Germany

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Overview

Purpose: To develop a rapid 2D HPLC method capable of fully characterizing a biologic formulation containing a mixture of a protein and a carbohydrate.

Methods: The biologic sample was first analyzed using reverse phase HPLC and UV at 280 nm to measure a surrogate protein bioformulation, bovine serum albumin (BSA) with a carbohydrate. A heart-cut was taken of the unretained carbohydrate and then analyzed in the second dimension while at the same time using HILIC HPLC for measurement of carbohydrate formulants with charged aerosol detection.

Results: The method enabled full characterization of a biologic formulation, including the active protein and an underivatized carbohydrate in just 23 minutes. Both methods are sensitive and precise, and with linear calibration. CAD was found to be five times more sensitive than UV at 280 nm for protein determination.

Introduction

Many of today's pharmaceutical formulations use proteins and other biotechnological APIs. These newer formulations make use of a variety of different excipients[1,2,3] including: surfactants, amino acids, salts, and carbohydrates. Carbohydrates are used to assist in the stabilization of the proteins in solution, the amount used being dependent upon the nature of the protein product requirements. A variety of carbohydrates can be used including sugars (glucose, sucrose, lactose, maltose, and trehalose) and 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. This would require a protein precipitation by acetonitrile before analysis. Another, reverse phase method would have to be run for the protein analysis.

To improve laboratory efficiency with faster analysis times and less sample preparation, the two methods were combined into a 2D heart-cut HPLC method so that the protein was separated from the sugars using reverse phase chromatography, where the sugars are not retained. Instead of precipitating the protein from the sugars for HILIC analysis, the C18 column performs the separation. Reverse phase is highly aqueous to begin, where the sugars elute, so the sugars were taken from the C18 void volume, transferred to a sample loop in combination with acetonitrile from the second pump, making it suitable for retention on the HILIC column. This allows for the combination of the two chromatography methods into a single system configuration as shown in Figure 1.

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 also used. The carbohydrate method used a Corona Veo SD, providing a sensitive and linear calibration of each of the seven, underivatized carbohydrates.

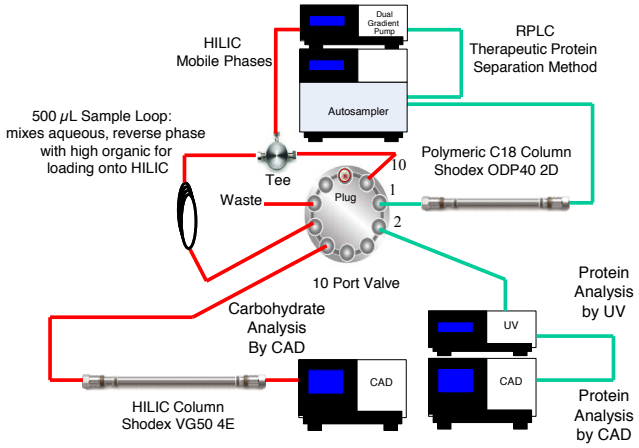

FIGURE 1. System schematic of 2D HPLC method development system using a sample loop between the RPLC and HILIC gradient elution methods.

Methods

Sample Preparations
Standards for BSA and each of the carbohydrates were dissolved in deionized water at 10 mg/mL and then diluted to 2000 µg/mL for the protein to which an equal volume of 2000 µg/mL of each carbohydrate solution was added. These stock solutions were then serially diluted with water down to 3.9 µg/mL.

Thermo SCIENTIFIC

Liquid Chromatography

HPLC System:	Thermo Scientific™ Dionex™ UltiMate™ 3000 DGP-3600SD, WPS-3000RS autosampler, and TCC-3000RS column oven with 2-position 10-port valve
HPLC Column 1:	Shodex™ Asahipak ODP-40 2D
HPLC Column 2:	Shodex™ HILICpak VG-50 4E
Column Temperature:	50 °C
Mobile Phase A1:	0.05 v/v-% Formic acid in water
Mobile Phase A2:	20 mM Ammonium acetate, 5 v/v-% Acetic acid in water
Mobile Phase B:	Methanol
Mobile Phase C:	Acetonitrile
Injection Volume:	2 µL
Detector 1A:	DAD, 214 nm and 280 nm
Detector 1B (Protein):	Corona Veo SD, Temp High, Power function= 1.40
Detector 2 (Sugars):	Corona Veo SD, Temp High, Power function= 1.15
Filter:	5
Data rate:	10 Hz
Flow Gradients:	

Pump 1

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

Pump 2

Time (min)	Flow Rate (m/min)	%A2	%B	%C	Left Valve	Comment
-5.00	0.50	5	0	95	1_2	Precondition
0.00	0.50	5	0	95		
0.90	0.50	5	0	95	10_1	MeCN to Loop
1.35	0.50	5	0	95	1_2	Gradient
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		

Data Analysis

All HPLC chromatograms were obtained and compiled using Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data Station, 7.2 SR 2 software.

Results

The method effectively separated the hydrophilic sugars from the protein on the reverse phase C18 column, which allowed for the retention of the protein for quantitation and protected the HILIC column used for the carbohydrate analysis. The use of a large transfer sample loop allows for the aqueous carbohydrate collected from the reverse phase column to be adjusted to high acetonitrile for analysis by HILIC.

Calibration, Sensitivity, and Precision

Standards of both BSA and carbohydrates from 3.9 to 1000 µg/mL were analyzed in triplicate. An overlay of chromatograms of the carbohydrates (in combinations for convenience), 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 reduce response, but the Shodex VG-50 column mitigates this effect by using hindered amines on the solid phase.[4,5] This provided better response for these carbohydrates.

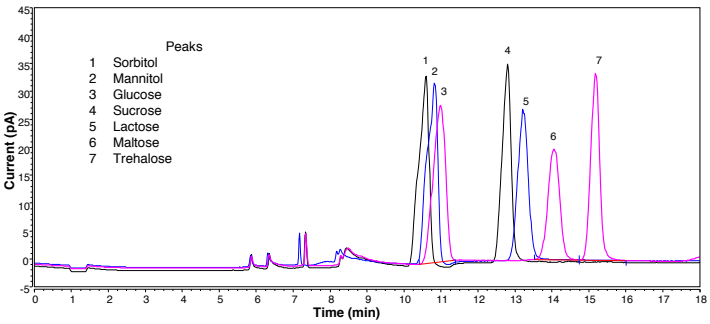

FIGURE 2. Seven carbohydrates by 2D-HPLC-CAD. All amounts of carbohydrates are at 2000 ng o.c.

A plot of the calibration data for five of the carbohydrates, with linear regression fits, is shown in Figure 3. The calibration curve for BSA by HPLC-CAD is shown in Figure 4, from amounts of 15.6 to 2000 ng o.c. All of the linear correlation coefficients, R^2, were greater than 0.997, as shown in Table 1.

The peak area percents for the triplicate injects were between 0.3 and 4.4%, with the greater values expectedly found closer to the limits of quantitation (LOQ), which are provided in Table 1. The LOQ values, calculated using the signal-to-noise ratio of 10, for the carbohydrates were between 14 and 29 ng o.c.

Also in Table 1 are the sensitivity and regression correlation values for the BSA, both by CAD and UV at 280 nm. Interestingly, for the BSA detection, the CAD technique provided a better signal-to-noise ratio than UV. For UV at 280 nm, the LOQ value was 79 ng o.c., and the LOQ value for CAD was 12 ng o.c., or over five times improved. This was attributed to due to better baseline stability and lower noise, as shown in Figure 5, with 125 ng o.c. of BSA analyzed by CAD and UV at 280 nm o.c. The baseline at 214 nm was much less stable (data not shown).

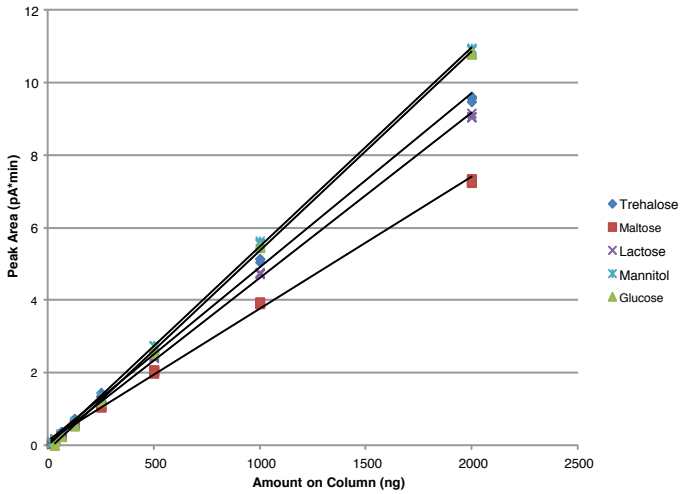

FIGURE 3. Linear calibration curves for four sugars by HPLC-CAD, 15.6 – 2000 ng o.c.

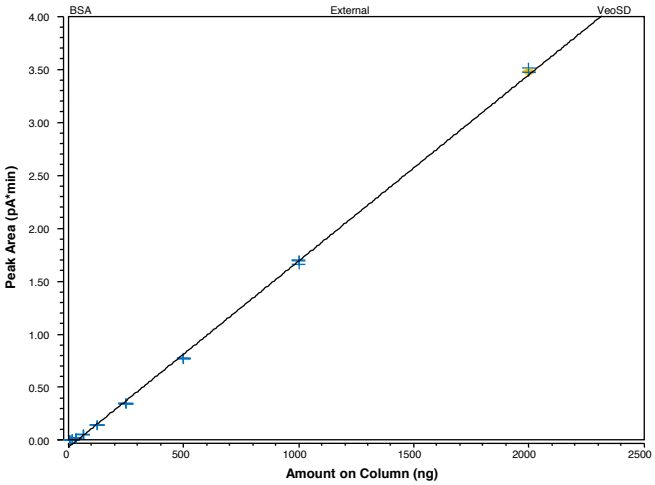

FIGURE 4. Linear calibration curves for BSA protein by HPLC-CAD, 5.6 – 2000 ng o.c.

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

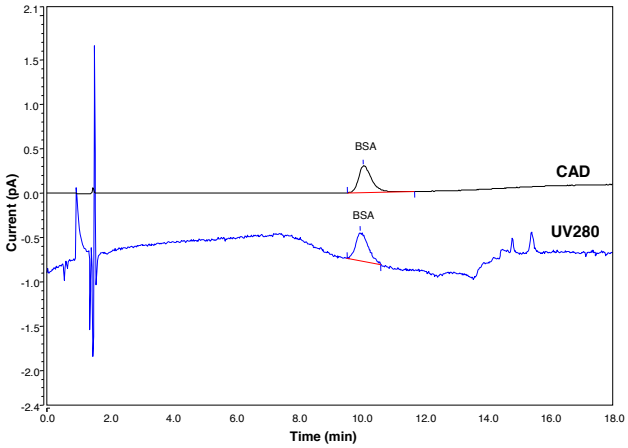

FIGURE 5. Overlaid HPLC chromatograms of BSA at 125 ng o.c. by UV at 280 nm (blue) and CAD (black).

Recovery

The 1000 ng o.c. solutions, of each BSA and a carbohydrate, were analyzed in duplicate and were used 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. These recovery values demonstrate that accurate results can be obtained from this fast and reliable method.

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

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 and useful workflow capable of characterizing a biologic formulation was developed. This approach enabled the simultaneous measurement of a protein and carbohydrate formulants in a bioformulation
- CAD is a universal detector and could be used to accurately determine underivatized carbohydrates and protein in bioformulations. Use in a QA/QC environment would save time, money, and the need for extensive sample preparation
- CAD was found to be over five-fold more sensitive for protein (BSA) than UV at 280 nm
- During method development two CADs were used for ultimate flexibility. However, the platform can be simplified by using just the UV detector for protein measurement, if sensitivity is not an issue

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