Intact Protein Analysis by RPLC Using Hand-Portable HPLC

Abstract

This application note demonstrates the use of the hand-portable Axcend Focus LC® system for intact protein analysis using reversed-phase liquid chromatography (RPLC). A mixture of five proteins ranging from 5.8 to 45 kDa was successfully separated using a capillary column in under 14 minutes, with high reproducibility and sharp peak shapes comparable to analytical-scale HPLC. The method shows that conventional HPLC protocols can be effectively adapted for use with the Axcend Focus LC by scaling flow rates, enabling robust, small-footprint analysis of proteins and other biomolecules in a compact format suitable for on-site or resource-limited environments.

Introduction

Biopharmaceuticals are rapidly becoming more popular and in recent years have increased from just one, to currently seven of the top 10 drugs. Biopharmaceuticals are proteins and peptides, such as monoclonal antibodies, that are genetically engineered from living cells. The size of these molecules typically falls in the range of 2 to 2,000 kilodaltons (kDa). Compared to small molecule pharmaceuticals, biopharmaceuticals have more complex structures and more reactive groups which often lead to lower stability and chemical properties that are easily modified. As a result, the quantitation identification, heterogeneity, impurity content and activity of each batch of target biopharmaceuticals must be thoroughly investigated before release. Due to the complexities of proteins, there is a need for powerful analytical techniques for their characterization, among which capillary liquid chromatography (LC) is one of the most important.

Transferring from established LC methods to micro- and nano-flow capillary LC is a simple procedure if you follow these guidelines. The mobile phase flow rate must be significantly reduced; other separation conditions (i.e., stationary phase, temperature, mobile phase components and gradient program) remain nearly the same. If the ratio between column length (L) and particle size (dp) is kept within +50% and -25% (as referenced in the United States Pharmacopeia guidelines), then transferring the method should start with adjusting the mobile phase flow rate to keep the linear velocity unchanged. More specifically, flow rate should be scaled according to the change in cross-sectional area of the column, which is proportional to the square of the ratio of the column diameters.

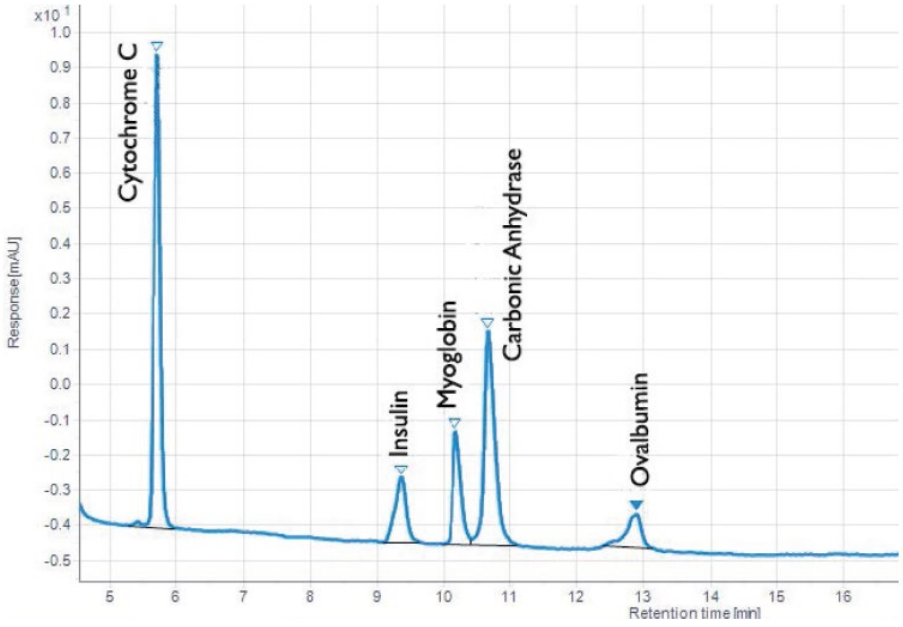
Materials and Methods

This brief application note demonstrates the use of small-footprint capillary HPLC by the Axcend Focus LC for intact protein analysis. Specifically, the separation of five test proteins in the range of 5.8 to 45 kDa is shown using reversed phase (RP) conditions. Other protein samples can be analyzed using the Axcend Focus LC merely by adapting established conventional high performance LC methods to the capillary column format.

Instrumentation	Axcend Focus LC w/UV Detector at 275 nm wavelength	
Column	CoAnn C4, 1.7 µm FPP, 300 A, 100mm	
Mobile phase A	0.1% TFA in water	
Mobile phase B	0.1% in acetonitrile	
Flow rate	1.25 µL/min	
Injection detail	0.2 µL	
Sample concentration	0.25 mg/mL carbonic anhydrase, myoglobin, human insulin, ovalbumin each and 0.1 mg/mL cytochrome C mixture in water	
UV detection wavelength	275 nm	
Column temperature	22° C	
Gradient	*Time (min)*	*Composition B (%)*
	0	5
	1	30
	6	40
	14	60
	15	95
	19	95

Results

5 Protein Test Compounds



Analyte	MW (kDa)	RT (min)	RT %RSD*	Tailing factor	Half height peak width (min)
Cytochrome C	12.3	5.70	1.92	1.06	0.11
Insulin	5.8	9.35	1.14	0.93	0.17
Myoglobin	16.7	10.17	0.70	1.67	0.13
Carbonic Anhydrase	30	10.67	0.39	1.28	0.16
Ovalbumin	45	12.89	0.85	0.68	0.21

(*n = 6)

Conclusion

- The Axcend Focus LC is well suited for the separation of biomolecules, such as peptides and proteins.
- Transferring methods from conventional to capillary LC should involve scaling the mobile phase flow rate according to the change in column cross section.
- Chromatographic results obtained using the Axcend Focus LC demonstrate reproducible separation of a mixture of five test proteins in the size range of 5.7 to 45 kDa within 14 min.
- Good peak shapes comparable to conventional HPLC are observed for all proteins.
- Improved resolution and greater peak capacity can be obtained by using longer capillary columns.