Increasing resolution of capillary LC reversed-phase analysis of antibodies and other proteins of biotechnical importance

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Introduction

Liquid chromatographic (LC) separation of peptides and small proteins has been successfully performed for at least two decades, while the reversed-phase (RP) separation of large biomolecules, such as antibodies, fusion proteins, and enzymes has experienced more modest development. Peptides act chromatographically much like small drug molecules and that empirical base has an extensive literature for the analyst to employ. The advent of biotechnology and the need for analytical techniques to examine protein-based therapeutic agents has resulted in the RP of large proteins being studied more carefully.

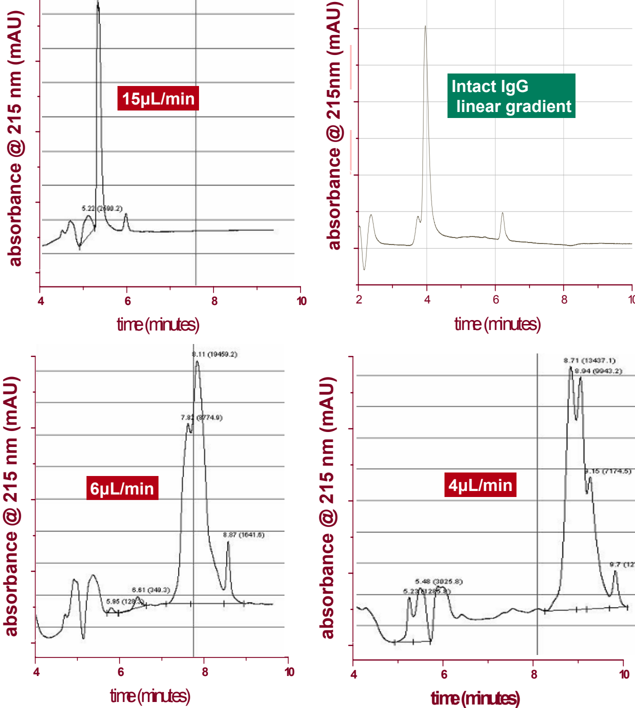
Experimental

Instrument: Eksigent ExpressLC-100 capillary liquid chromatograph, pictured below.
Column: Agilent SB300-C18, 0.3 x 150mm, 3.5µ particles
Column temperature: Ambient
Mobile phase:
linear gradients, A=water with 5% acetonitrile; B=acetonitrile with 5% water; both A and B containing 0.1% TFA; 100% A to 100% B in 10 minutes
step gradients, A=water with 5% acetonitrile; B=acetonitrile with 5% water; both A and B containing 0.1% TFA; 100% A to 100% B in 10 minutes, 1 minute steps starting at 1 minute
Flow rate: 4-15 µL/minute
Injection volume: 80-120 nL
Detection wavelength: 215 nm

ExpressLC-100 Capillary HPLC System

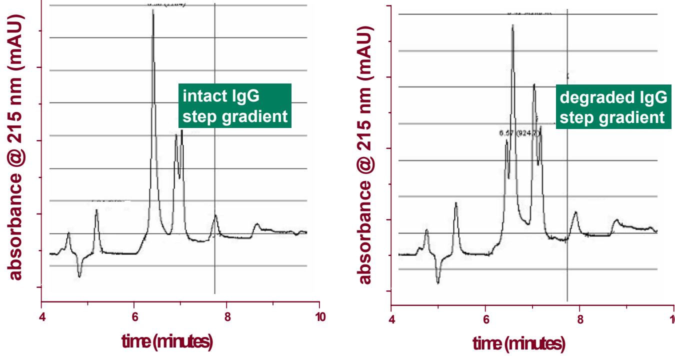
Dependence of isoform resolution on mobile phase flow rate

A conventional RP method is capable of determining the protein concentration and a retention time characteristic of the main species of interest. In the most favorable cases, the relative abundance of the various glycoforms and impurities can also be determined. For these reasons, the RP gradient determination has become a standard assay as part of most biologic drug species examination protocols. The RP separation of large protein molecules by conventional LC often requires very long (>60 minutes) analysis times to achieve reasonable peak shape and isoform resolution, since low (<0.7mL/min) flow rates are required. As the chromatograms at **15µL/min, 6µL/min, and 4µL/min** below demonstrate, IgG selectivity is increased in capillary LC in a similar fashion; however, the run times are relatively short.



Traditional linear gradient compared to step gradient for examining IgG degradation

The mechanism for the attachment, residence time, and release of large protein molecules in RP chromatography requires the mobile and stationary phases to be nearly in kinetic equilibrium. Conventional LC conditions required for analysis therefore often require very shallow gradients (in addition to slow mobile phase flow rates; discussed in the **previous section**) to allow analyte/stationary phase interaction. The chromatographic peak profiles are nonetheless often lacking in efficiency and selectivity. Apparently due to the very accurate gradient profiles, at least some proteins can be analyzed with more resolution than with a conventional **linear gradient** by use of **step gradients** (see experimental section for details). This is demonstrated below for both an **intact and a degraded polyclonal rabbit IgG sample.**



Conclusions

For capillary LC, it has been shown that for a high flow rate of 15µL/min, there is little chromatographic detail; as the flow rate decreases to 6µL/min and then to 4µL/min, more isoforms structure is apparent. In a second study, the precise gradient control and rapid re-equilibration allowed the use of multiple-step gradients. The result was increased isoform/impurity resolution when compared with standard linear gradients. Separations of an intact and degraded IgG via the two gradient modes were compared to illustrate the utility of step gradients in RP capillary LC analysis of antibodies..