W0930-04-21

# Fluorescence Detection by HPLC-FLD of Antibodies and Other Complex Proteins at Low Concentrations Utilizing Intrinsic Fluorescence for Reliable Protein Characterization

Sydney Huffman, Regan Allen, Jessica Kawakami, PhD and Rathna J. Veeramachaneni, PhD

KCAS Bio

**CONTACT INFORMATION:** Sydney.Huffman@kcasbio.com, kcasbio.com/contact-us

## PURPOSE

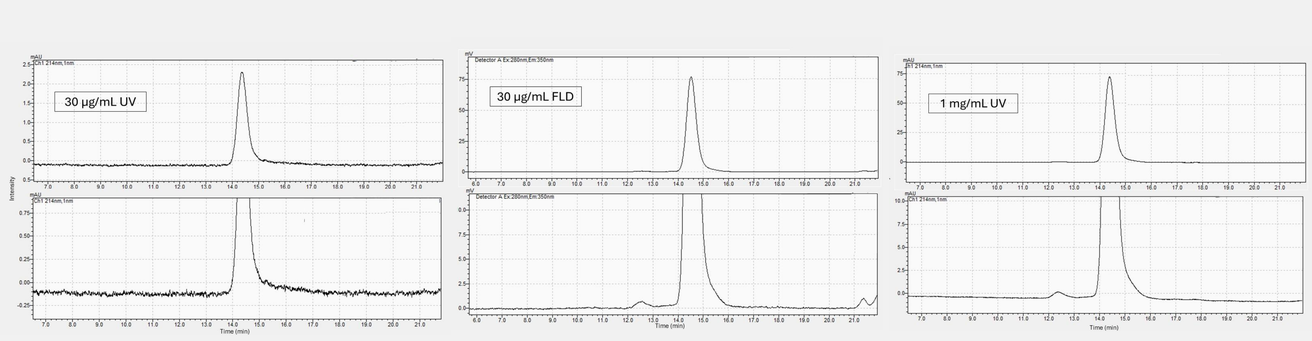
Detecting and quantifying antibodies and complex proteins in their monomeric form at low concentration levels cannot always be achieved using HPLC with UV detection. Sensitivity and resolution are some of the limitations to this approach that often leave pharmaceutical companies to decide between increasing the concentration range or exploring LC-MS/MS quantitation. The conventional notion is that fluorescence detection is solely used to analyze fluorescently tagged analytes. However, the intrinsic aromatic amino acid composition of 150 kDa antibodies enables fluorescence detection without the need for labeling. As proof of concept, we have developed and validated an HPLC-FLD method combined with size-exclusion chromatography (SEC) to effectively detect antibodies and monomeric species of complex proteins at low microgram/mL concentrations in formulations to determine concentration and antibody percent purity for generation of certificates of analysis (CoA).

## METHOD(S)

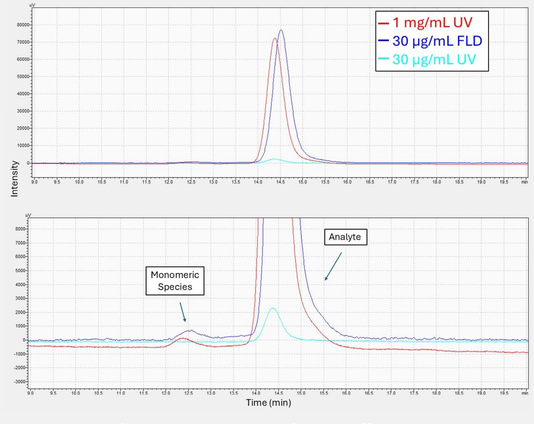
We developed a method for detecting and quantifying low concentration antibodies using the excitation and emission wavelengths of tryptophan and the same HPLC conditions we would use for an HPLC-UV method for the antibody. The aromatic-structured amino acids (tryptophan, tyrosine, and phenylalanine) fluoresce when excited by wavelengths in the ultraviolet range, as opposed to the visible range like other fluorescent tags. Samples were analyzed using SEC with isocratic flow for separation of the multimeric species. Samples were injected on a Shimdazu Nexera HPLC-UV/FLD system with a 7.8 x 300 mm, 5 µm column. Repeatability, linearity, accuracy, and specificity were evaluated.

## RESULT(S)

Using FLD, the antibody formulation prepared at microgram/mL concentrations yielded peak area responses comparable to those obtained with UV detection that needed 30-fold higher concentrations. This meant that 3% of the total sample is sufficient for FLD to achieve the same response as UV and without any additional sample prep needed. Additionally, the UV chromatogram did not detect any additional peaks due to the limited sensitivity and results in a percent area of 100%. The FLD chromatogram detects smaller, separated peaks for a better representation of the analyte purity. The peak area responses for FLD were linear, re-producible, and met GLP method validation acceptance criteria for repeatability, linearity, accuracy, and specificity. The method was used for determining the area percent purity of the antibody and a GLP certificate of analysis was generated and used for dosing and bioanalytical quantitation. The fluorescence detector is a low-cost alternative to mass spectrometry to achieve better sensitivity than a UV detector.




*Peak intensity comparison of the different detectors.*




*An overlay of the peak intensities for the different detectors.*

| Detector | Antibody Peak Area | Monomeric Species Peak Area | Total Peak Area | Area Percent Purity (%) |
|---|---|---|---|---|
| UV | 83,463 | Not detected | 83,463 | 100% |
| FLD | 2,284,590 | 27,997 | 2,312,586 | 98.8% |

## CONCLUSIONS

We have demonstrated a method for analyzing low concentrations of antibodies using fluorescence detection on UHPLC without fluorescent tagging. Due to the sensitive nature of a fluorescence detector when compared to a UV detector, this approach is advantageous for quick, re-producible, and low-cost methods for low concentrations of antibodies. The use of fluorescence detection for low concentration large molecule analytes is a simple swap that can detect antibodies and complex proteins in their monomeric form.

| Validation Parameter | Result | Pass/Fail |
|---|---|---|
| Repeatability | 0.6 - 0.9% RSD (n=6) | Pass |
| Linearity | Coefficient of Determination 1.00 | Pass |
| Accuracy | 98.6% - 100.3% | Pass |
| Limit of Quantitation | Concentration 30 µg/mL<br>S/N 887 – 1,206<br>3.3% RSD (n=9) | Pass |

## ACKNOWLEDGEMENTS

David Masters-Moore M.S., M.B.A.
No conflict of interest