Form-Q119

Standard Operating Procedure for Determination of Concentration of DFO-Protein by HPLC

1. **Purpose:** To describe the procedure to determine the mass concentration and chemical purity of DFO-Protein intermediate using HPLC.

2. **Scope:** The HPLC method described here will be used for determining the chemical purity and concentration of the DFO-protein intermediate. This result will be used for next step in the determination of protein amount present per dose of the ^{89}Zr-protein.

3. **Responsibilities:** Contract Sites & [Insert your institution name here]: To follow this procedure as written and to ensure that all results, control information, and raw data are documented as specified in dedicated Quality Control Laboratory Notebooks and recorded on the appropriate testing forms.

4. **References**
   - 4.1 USP 28 ‹621› Chromatography
   - **4.2 PBS-HPLC-L,** "Master Compounding Record for Preparation of Analytical HPLC Mobile phase for ^{89}Zr-Protein"

5. **Forms**
   - **Form Q119**, "Preparation of Protein Standard Solution and Dilutions Form"

6. **Policies**
   N/A

7. **Materials and Equipment**
   - **7.1.** Analytical HPLC Column Superdex TM 200 10/300GL, (GE Healthcare) or equivalent. (**SS-Super**)
   - **7.2.** High Performance Liquid Chromatography (HPLC) System consisting of a high-pressure pump, either an auto-sampler/injector, or manual injector; column and flow-through

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radioactivity detector; & either a variable or multi-wavelength UV detector, with data acquisition system.

7.3. DFO-Protein Standard, (**SS-DFO-Protein**)

7.4. **PBS-HPLC-L,** "Master Compounding Record for Preparation of Analytical HPLC Mobile phase for ^{89}Zr-Protein"

7.5. Automatic pipettors with disposable tips and glass graduated cylinders of appropriate sizes

7.6. Glass volumetric flasks and glass or plastic vials for dilutions

8. Procedures

8.1. Preparation of Standards

8.1.1. **A Stock DFO-Protein standard solution** consisting of DFO-Protein at 1 mg/mL concentration should be made as directed in **Form Q119**.

8.1.2. **Dilutions of the stock DFO-Protein standard solution:** Several 10-fold to 2-fold dilutions of the stock standard solution are needed to obtain standard concentrations appropriate for the analysis. All stock standard solution dilutions should be made using the HPLC mobile phase (**PBS-HPLC-L**). Use **Form Q119**, "Preparation of DFO-Protein Standard Solution and Dilutions Form," to prepare the standard concentration appropriate for analysis.

8.2. Calibration Curves

8.2.1. Inject a minimum of five dilutions of the standards so that the concentrations measured in the standard curve either bracket the solution to be tested or are all greater than the sample to be tested. The lowest standard to be tested must be less than the maximum allowed. For example, if the injected dose is 5 mL then the minimum acceptable limit, for a 5 mL injection is 1 mg/5 mL i.e. 0.2 mg/mL. The lowest concentration standard should be < 0.02 mg/mL and the concentration (UV absorbance or Mass peak area) of the highest standard must be greater than that for the product sample injection.

8.2.2. The retention time for DFO-Protein is approximately 16 minutes. Sample chromatograms are shown in Figures 1. All standards must be baseline resolved (resolution > 1.5) for a valid analysis.

Parameters

UV Detector: **Set to 280 nm**

Flow Rate: **0.8 mL/min**

Eluent: **PBS-HPLC-L**

Temperature: **Room Temperature**

Column: **Superdex TM** 200 10/300GL, (**SS-Super**)

8.3. Preparation and Analysis of Sample

8.3.1. A blank injection has been finished before the sample injection to make sure that no UV impurities from the column or/and the instrument.

8.3.2. Inject the sample. (This must be done in triplicate).

8.3.3. The test for release will be the completion of the standard curve and the first sample injection meeting criteria for release and the identity test. (A reference standard curve can be used if the investigational site has documented consistency of the standard response curve and a single standard run on the day of the test falls correctly on the standard curve). Two more injections will follow to assure the intermediate DFO-Protein chemical analysis obtained from the first injection was correct.

8.4. Data Analysis

8.4.1. Integrate all of the UV responses to obtain the peak areas for the DFO-Protein.

8.4.2. Determine the equation for the line of regression between the UV responses (area under the curve for the eluted peak) versus concentration.

8.4.3. Determine the correlation coefficient for the linear relationship.

NOTE: If the correlation coefficient is < 0.8, the standards must be re-run and an OOS investigation initiated.

8.4.4. Perform the reverse correlation to determine the concentration of DFO-Protein after the first coupling reaction.

8.4.5. Any other 280 nm UV absorbing chromatographic peaks that elute should be integrated individually and shown to sum to less than "5%" in the final product. Assume that the molar UV absorption coefficients for the unknown contaminants are the same as for DFO-Protein and that the DFO-Protein standard curve is applicable to the unknown contaminants.

ACCEPTANCE CRITERIA

HPLC Purity: **≥ 95%**

Sample chromatograms are presented in Figures 1. Figure 1 shows an UV absorbance from a chromatogram of DFO-Protein standard. The chromatograms in figures 1 used the methods including mobile phase, **PBS-HPLC-L**, and **Superdex TM** 200 10/300GL column described in this procedure.

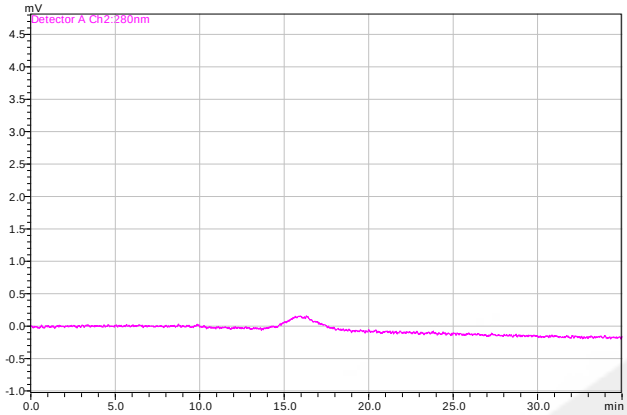

Figure 1. This figure shows the elution/purity of the DFO conjugated panitumumab from Amgen (10 µL injected of a 26 µg/mL solution), using PBS-NaN3-NaCl (**PBS-HPLC-L-**) mobile phase and the Superdex 200 10/300 GL column with a flow rate of 0.8 mL /min. This chromatogram shows that, although small, the minimum standard concentration needed < 26 µg/mL can be detected with sufficient signal to noise for quantitation of the UV absorbance.

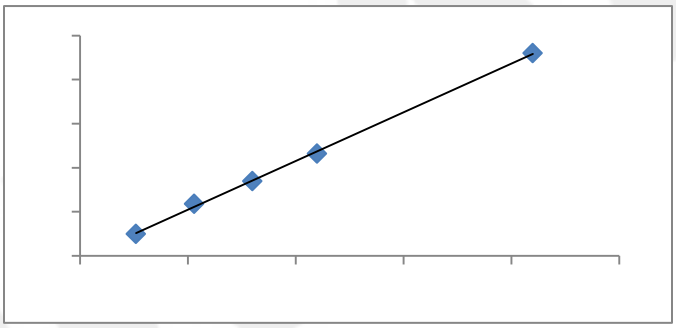

Figure 2. Standard calibration curve obtained in the different concentrations (26 µg/mL – 210 µg/mL) of DFO-panitumumab. X-axis is for concentration (mg/mL) and Y-axis is for normalized UV peak area (Y X 10^5) of HPLC chromatograms.