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Immunoglobulin and Albumin SE-HPLC

Determination of Molecular Size Distribution of Immunoglobulins and Albumins by SE-HPLC

Purpose and Scope

This document is to provide a method to determine molecular size distribution of plasma-derived immunoglobulin and albumin in non-specific immunoglobulin and albumin preparations (eg., Normal Immunoglobulin, Intragam P, Sandoglobulin, Octagam, Gamunex, Albumex etc.) and specific immunoglobulin preparations (e.g., WinRhoSDF, Rh(D), Imogam Rabies, Tetanus, Hepatitis B, Zoster, etc.) that comply with the BP/ Ph.Eur. monographs for Human Normal Immunoglobulins and Human Normal Immunoglobulins for Intravenous Administration.
The method is a fixed procedure (albeit constantly evolving), not a guideline, and is designed to ensure that all procedures including quality control, validity, record keeping, accountability and safety are adhered to.

Responsibility

The review of this method is the responsibility of the Biotherapeutics Director or their delegate. Reviews will be recorded on the Quality System. The amended method is then circulated to the relevant staff.

Background

The separation achieved by size-exclusion HPLC (SE-HPLC) is based on partitioning of molecules according to their size as they pass through the chromatography matrix. Large molecules, such as aggregates or polymers, being unable to enter pores of the matrix, are not retained and, hence, elute first. Smaller dimers, followed by monomers and then fragments, elute later as they are able to partly diffuse into the pores, depending on their size. The separated fractions are detected and quantified by photometry at 280 nm.

References

- IgG SE-HPLC validation report - stored electronically in Trim: *2010/005326 - THERAPEUTIC ADMINISTRATION - POST MARKET - Testing - Biochemistry Section - Assay Specific Data - IgG SE-HPLC – OLSS*
- Human Normal Immunoglobulin Monograph and Immunoglobulin for intravenous administration 01/2012:0918. *In: European Pharmacopoeia (8th Edition)*, Strasbourg, France: Council of Europe; 2016
- Human Albumin Solution Monograph 01/2013:0255. *In: European Pharmacopoeia (5th Edition)*, Strasbourg, France: Council of Europe; 2016
- Sandberg E, Daas A and Eposito-Farese ME. Collaborative study for the establishment of the human immunoglobulin biological reference preparation batch No. 2 *Pharmeuropa Special Issue*, BIOLOGICALS 2001;(1):25-42

WHS Requirements

Biotherapeutics HPLC procedures are covered by risk assessment forms 141213952012 (HPLC), 571292612003 (Sample preparation), and 12162532004 (Blood product testing).

All work in the Laboratory must be conducted according to the standards AS/NZS 2243.1: 2005 *Safety in laboratories* and the "Laboratory Safety" section of the Laboratory Operations Manual (LOM) on the QMS. In brief:

- Wear appropriate Personal Protective Equipment
- Label and classify all chemicals and reagents appropriately and use in accordance with the applicable Safety Data Sheets
- Handle all cells in accordance with *OGTR Physical Containment Level 2* (PC2) requirements and dispose of and record all genetically modified cell lines appropriately.
- Be aware of the specific requirements for the handling of glassware and sharps, potential biological hazards, animals and working alone.

Risk Assessments for the relevant activities are located at http://tgalqs/riskassess/index.htm.

Uncertainty of Measurement

Refer to the LOM for details regarding Uncertainty of Measurement.

Method

Materials/Equipment

Equipment

- HPLC equipped with a DAD (Diode array detector)
- SE Chromatography column – hydrophilic silica gel for chromatography R, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10,000 to 500,000 are suitable,

- TSKgel 3000SW, 600x7.5 mm (Cat#: 05103), or
- TSK gel 3000 SWxl 300x7.8mm (Cat#: 08541)

- Sample Vials – 2 ml Clear ABC Screw Top Vial (Supelco Cat No. 27329) with ABC Screw Cap with PTFE/Silicone Septa (Supelco Cat No. 27558).
- Filter Unit (Ground-joint Flask and Glass Funnel) and 0.20µm Nylon Membrane, 47mm diameter or equivalent
- Various micro-pipettes for reference and sample preparation.

Software

- Empower software (current version used by Biotherapeutics)

Materials

The following materials should be prepared as described in the Monograph – Liquid Chromatography. Some procedures, where relevant, can also be found in Chem-SOP-4 – General Procedures for HPLC and UPLC Analysis

Sodium Chloride Solution (0.9%)

- 0.9 g sodium chloride, NaCl
- dissolve in 100 mL of distilled water

Mobile Phase

- 4.88 g di-sodium hydrogen orthophosphate dihydrate, $Na_2HPO_4.2H_2O$
- 1.74 g sodium dihydrogen orthophosphate monohydrate, $NaH_2PO_4.H_2O$
- 11.69 g sodium chloride, NaCl
- dissolve in 2000 mL of distilled water, unadjusted pH should be about 6.9

Column Wash Solution A

- 17.76 g sodium sulphate, Na_2SO_4
- dissolve in 200 mL of distilled water, adjust to pH 3.0 with diluted sulphuric acid (H_2SO_4, 1:20 dilution), and make up to 250 mL with distilled water

Column Wash Solution B

- 1.22 g di-sodium hydrogen orthophosphate dihydrate, $Na_2HPO_4.2H_2O$
- 0.44 g sodium dihydrogen orthophosphate monohydrate, $NaH_2PO_4.H_2O$
- dissolve in 200 mL of distilled water
- add 50 mL of acetonitrile

Column Storage Solution (20% Ethanol)

- 200 mL ethanol
- 800 mL distilled water

Reference and Samples

Lyophilised reference preparation/sample must be brought to room temperature before reconstitution and is to be reconstituted according to the **manufacturers' instructions**. Reconstituted reference preparation/sample is then diluted with 0.9% sodium chloride to a final concentration of **40 mg/mL IgG**. Injection volume is 10 µL which corresponds to 400 µg protein load. (BP 2014: 50 – 600 µg protein).

Reference Material

EDQM, European Pharmacopeia Commission - Human Immunoglobulin (molecular size) BRP Batch 1.2

- Assigned potency = 700 mg/vial.
- Stored at +2°C to +8°C
- Reconstitute the contents of each vial with 7.0 mL of sterile ddH_2O to give a concentration of 100 mg/mL IgG
- Leave at room temperature for at least 2 hours until complete dissolution is achieved. Complete dissolution must be checked visually
- The reconstituted reference preparation may be stored at +4°C for a maximum of 14 days
- Frozen aliquots of reference preparation may be stored at -80°C for 36 months

Sample

After the initial sample receipt procedure has been completed by the sample receipt officer, the sample (including LIMS number) is stored **as per manufacturer's instructions**.

Official testing should only involve **fresh** samples**.**

Procedure

- Prepare the solutions and reference preparation/samples as described in **Materials**.
- Prepare and run the column
  - Create a new project or use an existing project if an appropriate one already exists, e.g., Intragam IgG
- For IgG SEC Instrument parameters see Table 1.0
- Create a second instrument method running distilled water through the column at 0.1 mL/min, set this as the Shutdown Method.
- For sequence set-up see Table 2.0, reference material injections can bracket up to 3 samples

Table 1 - Instrument Settings

Item	Description			
Pump Mode	Isocratic 100% - SEC Mobile phase			
Flow Rate	0.5 mL/min			
Maximum Pressure Setting on column	Dependent on column • TSK gel 3000SW, 600x7.5 mm (Cat#: 05103) set for 50 bar • TSK gel 3000SWxl, 300x7.8mm (Cat#: 08541) set for 70 bar			
DA Detector	Sample (nm)		Reference (nm)	
	Wavelength	Bandwidth	Wavelength	Bandwidth
	280	16	360	100
Auto injector and Heater	4°C			
Column Heater	21°C			

Table 2 - Sequence set-up

Vial Order	Sample name	# of injections
Vial 1	Blank (diluent solution)	1
Vial 2	Reference Material	4
Vial 1	Blank (diluent solution)	1
Vial 3	Sample 1	3
Vial 4	Sample 2	3
Vial 5	Sample 3	3
Vial 2	Reference Material	3
Vial 6	Sample 4	3
Vial 2	Reference Material	3
Vial 1	Blank (diluent solution)	1

Analysis

Analysis should be performed using the Empower Software. An electronic version of the Empower Manual can be found in the software.

Example of expected peaks (for IgG):

In the chromatogram obtained with the reference preparation, the principal peak corresponds to the IgG monomer. The monomer peak is preceded by a peak corresponding to the dimer. Any peak with a retention time less than that of the dimer corresponds to polymers and aggregates. Peaks with retention time longer than that of the monomer correspond to fragments.

Figure 1 - Human IgG BRP Chromatogram

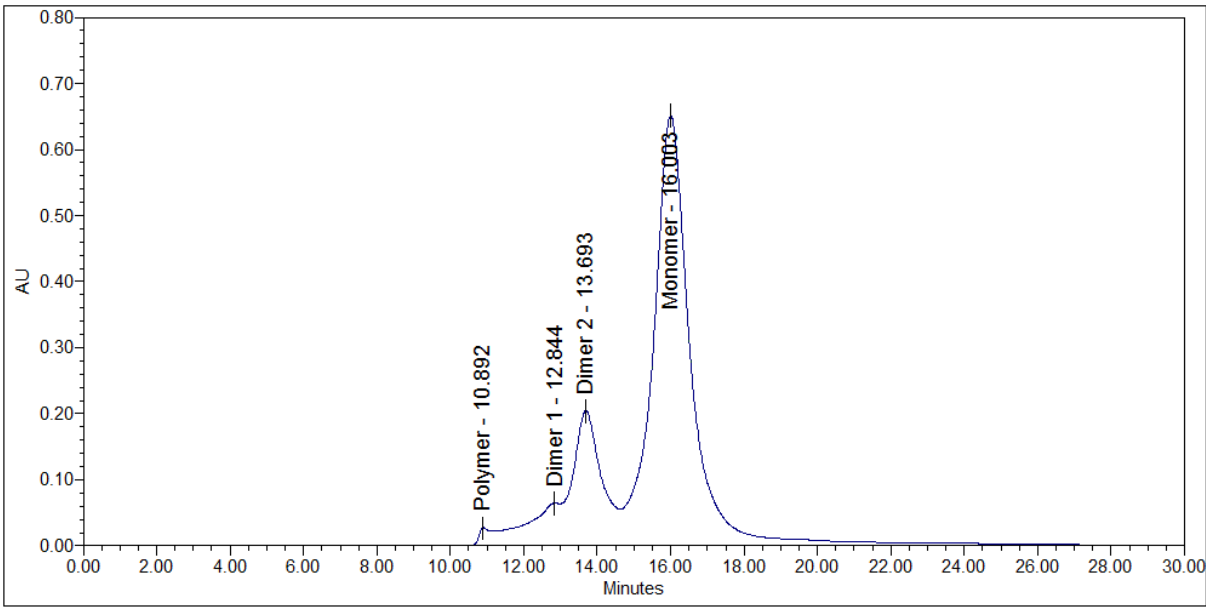
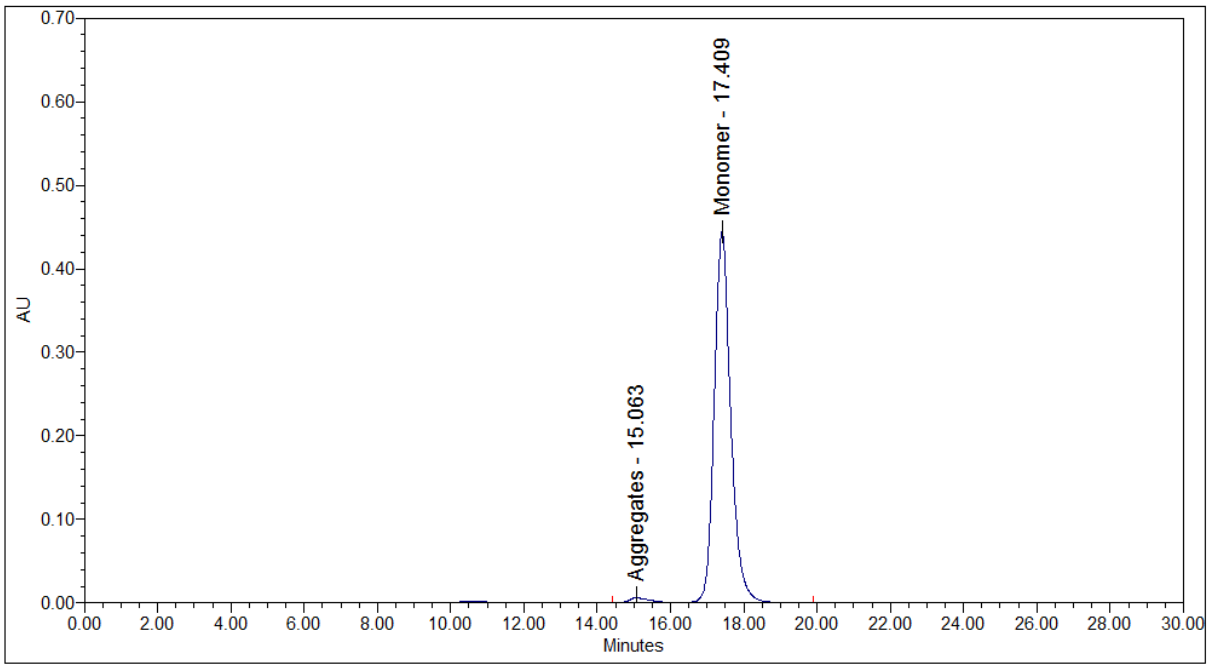


Figure 2 - Human Albumin Chromatogram



Results of a run can only be accepted when the following parameters are met:

System Suitability (assessed from IgG standard injections)

- The relative retention time of the dimer peak to the monomer peak of the reference preparation should be about 0.85
- The percent RSD of the peak area for the monomer peak of the reference preparation from triplicate injections at the start and at the end, bracketing samples, should be ≤ 2%

Sample Suitability

- The relative retention time of the dimer and monomer peak of the sample, relative to the corresponding peaks of the reference preparation should be 1 ± 0.02 (for IgG only)
- The percent RSD of the peak area for the monomer peak of the sample from triplicate injections should be ≤ 2% (for both IgG and albumin)

If any analyses do not conform to the system suitability criteria consult with supervisor.

When results that appear out of specification are observed, follow the procedures outlined in the LOM, Section 6, 'Testing Program'.

Saving and Storing Data

- Save electronic data into the relevant Trim file
- Scan any hard copies of worksheets, reagent preparation sheets or analysis results into the relevant file in trim
- Fill in appropriate column usage log

Column Care

NB: For a TSK gel 3000 SWxl (300 mm) column: 1 column volume (CV) is 15 mL, for a TSKgel 3000SW column (600 mm) 1 x CV is 30 mL.

At the end of each run, the column should be thoroughly cleaned prior to storage, as follows:

- Do not connect the column to the detector during cleaning
- Run the column at half the maximum recommended flow rate (e.g., ≤ 0.25 mL/min)
- Wash with 3 x CV of Column Wash Solution A
- Rinse with 3 x CV of filtered distilled water
- Wash with 3 x CV of Column Wash Solution B
- Rinse with 3 x CV of filtered distilled water
- Rinse with 3 x CV of Column Storage Solution
- Store column in Column Storage Solution with the ends of the column tightly capped

Associated Documents

- Chem-SOP-4 – General Procedures for HPLC and UPLC Analysis
- Bio-BPC-Form-10 – General HPLC - Worksheet