THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

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European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

General chapters (methods of analysis) supporting individual monographs on Biotherapeutics

European Pharmacopoeia training session on Biologicals

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General chapters

- General methods and guidance texts
- Editorial convenience
- Not mandatory "per se"
- Useful tool when there is no monograph
- Part of the standard when referred to in a monograph

Overview of applicable general chapters – analytical techniques

Mass spectrometry (2.2.43)

Statistical analysis of results of biological assays and tests (5.3)

Capillary electrophoresis (2.2.47)

Absorption spectrophotometry, UV-Vis (2.2.25)

Electrophoresis (2.2.31)

Isoelectric focusing (2.2.54)

Total protein (2.5.33)

Immunochemical methods (2.7.1)

Size-exclusion chromatography (2.2.30)

Liquid chromatography (2.2.29)

Chromatographic separation techniques (2.2.46)

Amino acid analysis (2.2.56)

Quantification and characterisation of residual host-cell DNA (2.6.35)

Host-cell protein assays (2.6.34)

Peptide mapping (2.2.55)

Glycan analysis of glycoproteins (2.2.59)

Non exhaustive list

Nucleic acid amplification techniques (2.6.21)

Assay of human coagulation factors (2.7.4, 2.7.10-11, 2.7.18-19, 2.7.22)

Assays of interferons (5.6)

Microbiological and viral safety chapters

(…)

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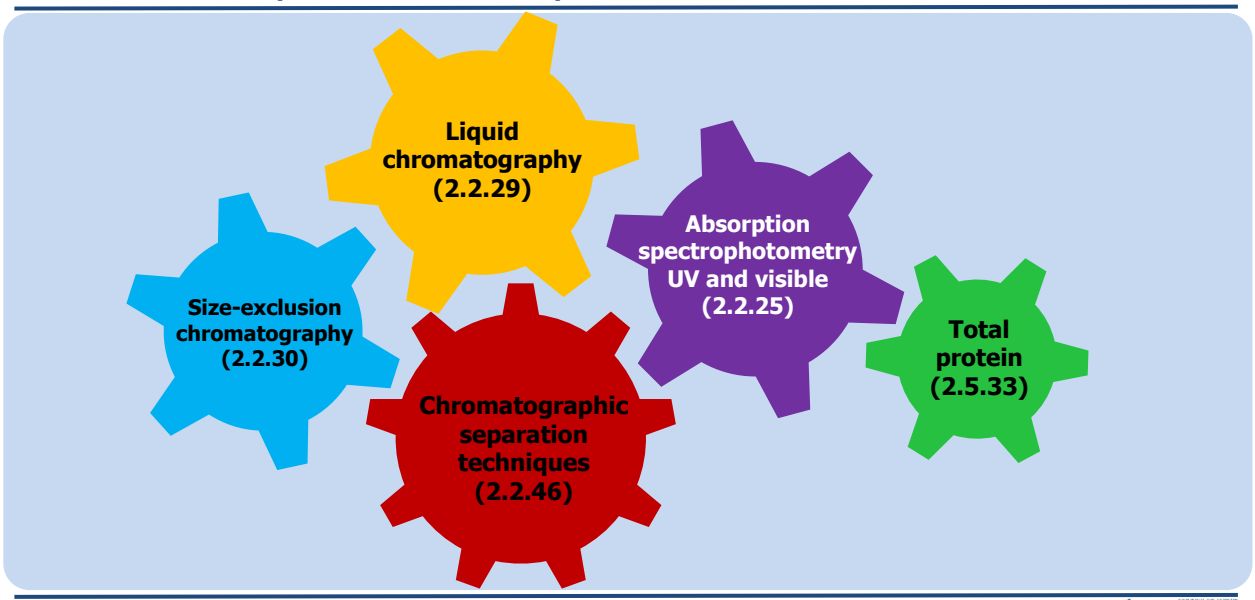
Assays of interferons (5.6)

Microbiological and viral safety chapters

(…)

General chapters – example of interconnections



Host-cell protein assays (2.6.34)

Quantification and characterisation of residual host-cell DNA (2.6.35)

Host-cell protein assays (2.6.34)

Table of content

2.6.34 HOST-CELL PROTEIN ASSAYS

This general chapter provides guidance for the development and validation of host-cell protein (HCPs) assay used to test products obtained by recombinant DNA technology. It does not exclude the use of alternative approaches that are acceptable to the competent authority.

1. Content

Host-cell protein assays (2.6.34)

Types of HCP assay

Process-specific assays (*also called product-specific HCP assays*)

- Developed and validated taking into account the specificity of the production process and using the host organism expressing the recombinant product
- Antigen derived from a mock run of the drug substance manufacturing process up to a step capable of generating sufficient quantities and broad spectrum of HCPs
- Antisera raised must cover a broad range of HCPs, in order to detect as many different HCPs as possible and also to accommodate process variations

Host-cell protein assays (2.6.34)

Platform assays

- Developed by individual manufacturers and customised for their processes and host organism
- Same sets of reference standards and reagents may be used to monitor HCPs in several products manufactured in the same host organism, provided that upstream processes (and downstream, if relevant) are sufficiently similar between these products

Generic assays

- Commercially available HCP test kits are commonly referred to as generic HCP assays
- They are intended to work broadly across similar expression hosts
- Detailed information on the preparation of the reagents may not be disclosed by the vendor

Host-cell protein assays (2.6.34)

Assay selection

- Risk assessment to support the choice between a generic, platform or a process-specific assay
- Takes into account the stage of development of the product, the nature of the host-cell and protein immunogenicity, the expression mode, the manufacturing process, and prior knowledge
- Assay lifecycle (e.g. reagent supply, consistency, assay validation, process change) to be considered

Host-cell protein assays (2.6.34)

Production and testing of the HCP Antigen

	Process-specific assays	Platform assays	Generic assays
Null cell line	- Derived from the same cell line	- Same host species across a company's portfolio	- HCPs may be derived from a combination of strains of an expression host species - May not mimic the process applied for the product of interest - *Detailed information may not be disclosed by the vendor*
Mock manufacturing process – *Upstream*	- Mimics the intended process - May be adjusted to cover worst case situations	- Mimics the platform upstream process that is used for several products	
Mock manufacturing process – *Downstream*	- Minimal processing recommended - Further processing could be considered		
Characterisation and testing	- Comparison of HCP population: mock vs intended process		

Host-cell protein assays (2.6.34)

Production and characterisation of the anti-HCP antibody reagent

	Process-specific & Platform assays	Generic assays
Immunisation	- Animal species: host that yields sufficient amounts and diversity of HCP-specific IgG - **Aim: immune response against both strong and weak antigens**	- No recommendation (steps carried out by the kit vendor)
Purification and preparation	- Protein A- or protein G-chromatography and/or HCP antigen affinity chromatography - Removal of aggregates may be required	
Characterisation and testing	- **Demonstration of coverage**: comparison immunostain vs total protein stain by 2D electrophoresis	- As for process-specific and platform assays, however limited control over reagent lot-to-lot consistency (comparative lot testing required)

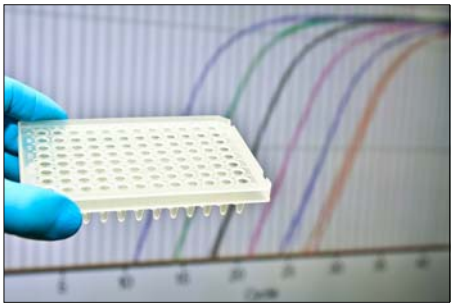
Host-cell protein assays - quiz

Can I use MS in complement to the ELISA immunoassay to characterise individual HCPs?

Chapter 2.6.34 focuses on the ELISA immunoassay (most widely used HCP assay format) but does not exclude the use of alternative approaches. The use of orthogonal methods (e.g. electrophoresis, HPLC, WB, MS) to characterise the various HCPs is recommended to support the development and selection of the assay.

Quantification and characterisation of residual host-cell DNA (2.6.35)

- **Analytical methods** for residual DNA quantification, size characterisation in biologicals produced in cell substrates
  - Focus on most widely used techniques: **qPCR** and **Threshold method**
  - *Does not exclude the use of alternative approaches that are acceptable to the competent authority*



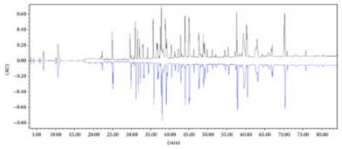
Peptide mapping (2.2.55)

Peptide mapping (2.2.55)

- Harmonised chapter

- Ongoing revision (Stage 2 – official inquiry; Pharmeuropa 26.4 & 29.4)

- Assistance in the development of peptide mapping and its validation



- Identification test for proteins; dedicated reference standard

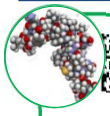
Peptide mapping (2.2.55)

Peptide mapping (2.2.55)

Isolation and purification	Selective cleavage of peptide bonds	Chromatographic separation	Analysis and identification of peptides
	• Cleavage agents examples • Pre-treatment of sample • Pre-treatment of the cleavage agent • Pre-treatment of the protein • Establishment of optimal digestion conditions	• Chromatographic column • Solvent • Mobile phase • Gradient • Isocratic elution • Other parameters • **Validation**	Use during development in support of regulatory applications

Experimental means for measuring the method performance; guidance on SST requirements

Peptide mapping in monographs



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TERIPARATIDE

Teriparatidum

(...)

IDENTIFICATION

A. Peptide mapping (2.2.55).

SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS

(...)

CHROMATOGRAPHIC SEPARATION

Liquid chromatography (2.2.29). *Store the solutions at 2-8 °C and use them within 72 h.*

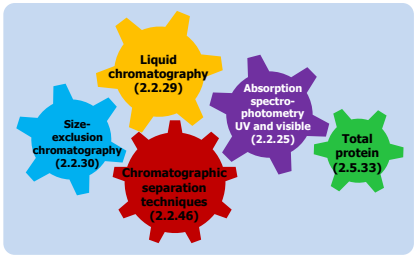
(...)

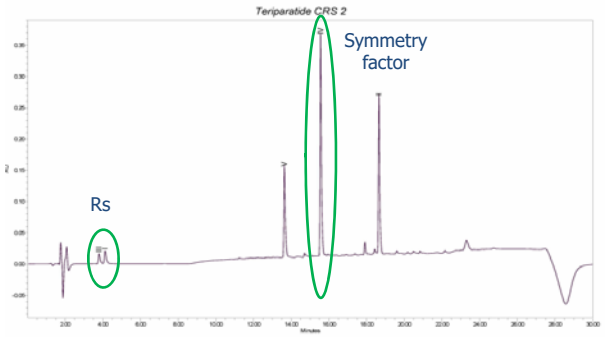
System suitability:

- the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of teriparatide digest supplied with *teriparatide CRS*;
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II, III, IV and V:

  symmetry factor: maximum 2.3 for the peak due to fragment IV;

  resolution: minimum 1.5 between the peaks due to fragments I and III.





Peptide mapping - quiz

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INSULIN, HUMAN

Insulinum humanum

(...)

IDENTIFICATION

(...)

B. Peptide mapping (2.2.55).

SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS

Test solution. Prepare a 2.0 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid* and transfer 500 µL of this solution to a clean tube. Add 2.0 mL of *HEPES buffer solution pH 7.5 R* and 400 µL of a 1 mg/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 mL of *sulfate buffer solution pH 2.0 R*.

Can I use any *Staphylococcus aureus* V8 protease?

Protease defined in the reagent description must be used.

***Staphylococcus aureus* strain V8 protease, type XVII-B.** *1115800.* [66676-43-5].

Microbial extracellular proteolytic enzyme. A lyophilised powder containing 500 units to 1000 units per milligram of solid.

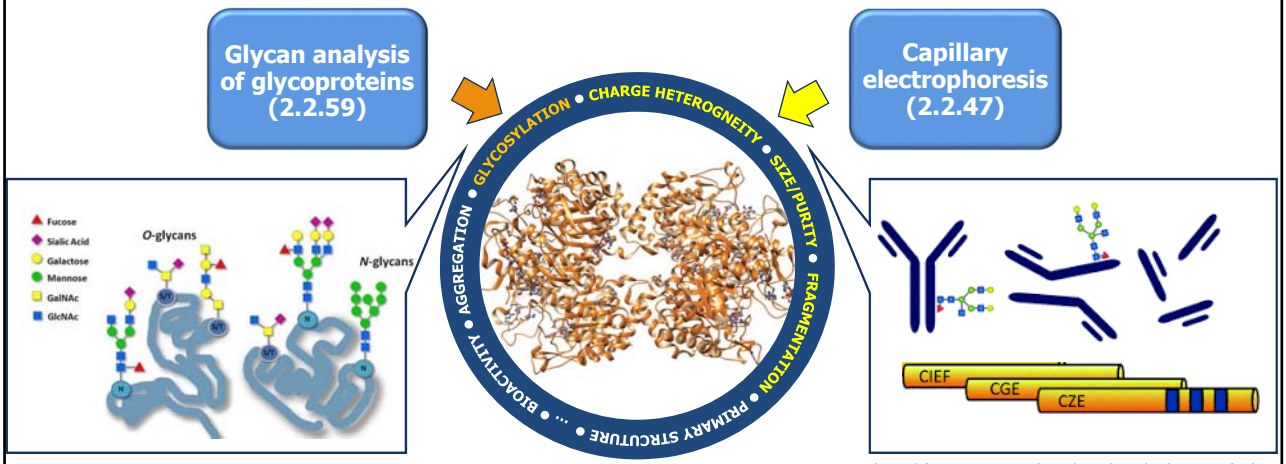
Information on enzyme found suitable during the laboratory verification are supplied in Knowledge Database.

Knowledge Database edqm

Test(s)	Brand Name/Information
Identification B, peptide mapping	Staphylococcus aureus strain V8 protease R ; Endoproteinase Glu C, EC 3.4.21.19, SIGMA P6181, column : SpherisorB ODS 11

....other analytical procedures used for evaluation of quality attributes of biotherapeutic proteins...

Tools for Analytical Characterisation/Quality Control



Adapted from Lunte *et al.* Analytical Methods, 2014 (15)

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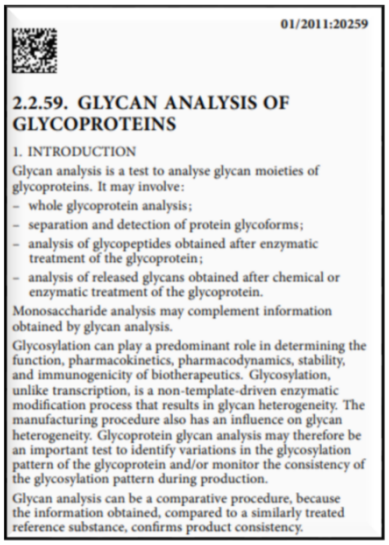
Glycan Analysis of Glycoproteins (2.2.59) (1)

- Describes different approaches used for glycoprotein glycan analysis and requirements for the application of methods and validation of methods.
- Provides framework and guides analysts in the choice of appropriate procedures -- spread of methods suitable for almost all products.
- Provides links to other general chapters relevant to the analysis of glycosylation, *e.g.* at the level of intact glycoprotein or cleaved glycan chain (CE (2.2.47; MS (2.2.43); SEC (2.2.30); IEX (2.2.46); IEF (2.2.54)).
- Glycan analysis is not a single general method, but involves the application of specific procedures and the development of specific glycan maps for each unique glycoprotein.

⇨ Specific procedures are therefore indicated in relevant specific monographs.



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2.2.59. GLYCAN ANALYSIS OF GLYCOPROTEINS

1. INTRODUCTION

Glycan analysis is a test to analyse glycan moieties of glycoproteins. It may involve:

- whole glycoprotein analysis;
- separation and detection of protein glycoforms;
- analysis of glycopeptides obtained after enzymatic treatment of the glycoprotein;
- analysis of released glycans obtained after chemical or enzymatic treatment of the glycoprotein.

Monosaccharide analysis may complement information obtained by glycan analysis.

Glycosylation can play a predominant role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of biotherapeutics. Glycosylation, unlike transcription, is a non-template-driven enzymatic modification process that results in glycan heterogeneity. The manufacturing procedure also has an influence on glycan heterogeneity. Glycoprotein glycan analysis may therefore be an important test to identify variations in the glycosylation pattern of the glycoprotein and/or monitor the consistency of the glycosylation pattern during production.

Glycan analysis can be a comparative procedure, because the information obtained, compared to a similarly treated reference substance, confirms product consistency.

Glycan Analysis of Glycoproteins (2.2.59) (2)

Glycan analysis procedures

Analysis of intact glycoprotein

- Mass spectrometry
- Isoelectric focusing
- Capillary electrophoresis
- Ion-exchange chromatography
- Size exclusion chromatography
- SDS-PAGE

Analysis of glycopeptides

- Proteolysis

- Direct analysis by MS
- Separation by LC or CE prior to direct analysis by MS
- Deglycosylation of the glycopeptides

Analysis of released glycans

- Release (enzymatic*/ chemical of glycans):
  - analysis of unlabelled glycans (HPAEC-PAD)
  - analysis of labelled** glycans (LC, CE, MS)

*Examples of enzymatic cleavage agents
** Examples of fluorescent labels and suitable techniques

Monosaccharides analysis

- Colorimetric methods (quantification of specific classes, *e.g.* sialic acids…)
- Separation methods (overall monosaccharide composition):
  - mild H^+ hydrolysis or enzymatic treatment;
  - HPAEC-PAD;
  - fluorophore labelling, RP-HPLC, IEX or CE

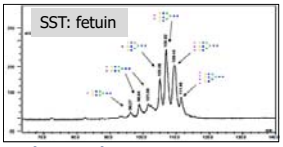
Glycan Analysis of Glycoproteins (2.2.59) (3)

Evaluation and analysis of data

- confirmation of identity of individual structures or families of structures (MS);
- confirmation of compliance of the substance being tested with qualitative requirements (*e.g.* retention times; comparison with process or system suitability RS)
- confirmation of compliance of the substance being tested with quantitative requirements (*e.g.* by reference to a RS (e.g. sialic acid); normalisation procedure; *Z* number).

Reference standards

- verification of suitability of the system:
  - a reference substance for the substance tested;
  - glycan moieties liberated from:
    - a fully characterised reference standard of the substance tested;
    - from glycoproteins (*e.g.* **fetuin**, IgG);
  - glycan markers.



- confirmation that the article under test complies with specified requirements:
  - the reference standard is a preparation of the substance being tested.

Glycan Analysis of Glycoproteins (2.2.59) (4)

The following monographs include reference to chapter 2.2.59:

"Use a suitable method developed according to general chapter 2.2.59. Glycan analysis of glycoproteins, section 2-3."

- Alteplase for injection (1170)
- Etanercept (2895)
- Follitropin (2285)
- Follitropin concentrated solution (2286)
- Human coagulation factor IX (rDNA) concentrated solution (2522)
- Human coagulation factor VIIa (rDNA) concentrated solution (2534)
- Infliximab concentrated solution (2928)

chapter 2.2.59 becomes mandatory

Glycan Analysis – Quiz

QUESTION: How do I apply chapter 2.2.59 to my preparation (*i.e.* a recombinant DNA protein not covered by an individual monograph)?

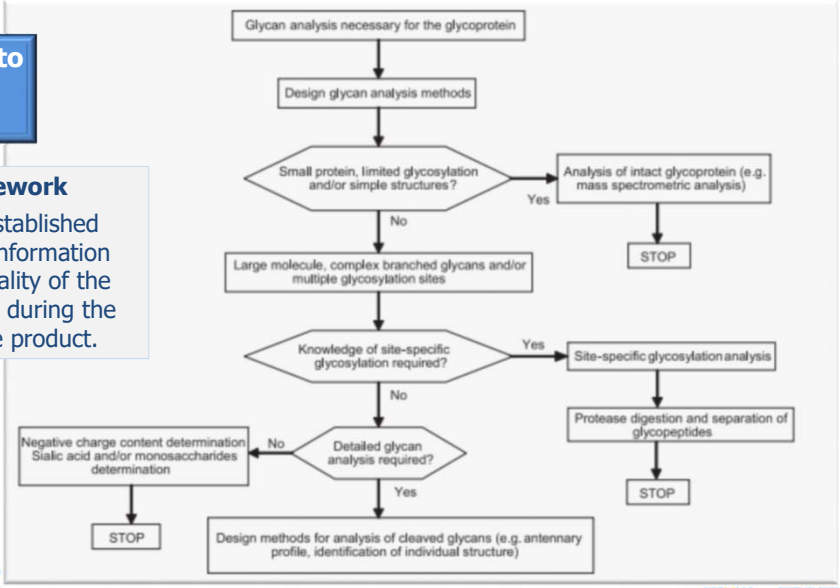
RESPONSE:

- The preparation complies with the requirements given in **Ph. Eur. general monograph on *Recombinant DNA technology, products of (0784).***
- General chapter 2.2.59 provides means for measuring the **overall performance of the glycan analysis method during development**:
  - extent of method development and analytical validation is selected on the basis of their suitability for a specific product → **points to consider during method development**:
    - isolation and purification (or desalting) of the glycoprotein;
    - enzymatic (or chemical) treatment of the glycoprotein to selectively release *N*- or *O*-linked glycans
    - verification of released sialic acid and monosaccharide residues;
    - chromophore labelling of the released glycans;
    - glycan identification and quantification (*e.g.* determination of the *Σ* number);
    - determination of site occupancy (relative quantities of glycosylated and non-glycosylated peptides)'
    - .....

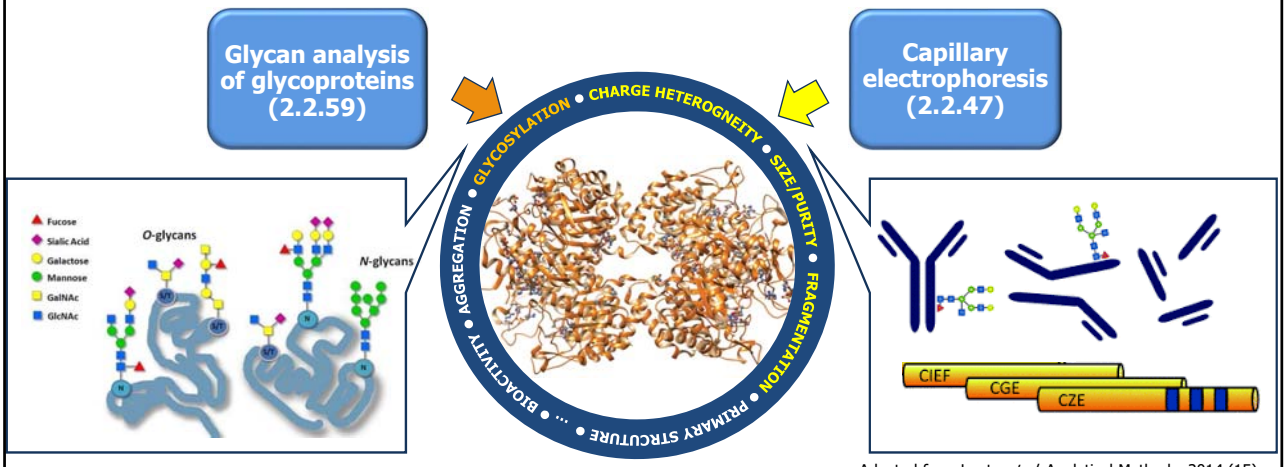
Glycan Analysis of Glycoproteins (2.2.59): Summary

Guidance on methods to be used when glycan analysis is required

- **Decision-making framework**
- Choice of procedures is established according to the level of information required to ensure the quality of the glycoprotein and is set up during the development phase of the product.



Tools for Analytical Characterisation/Quality Control



Adapted from Lunte *et al.* Analytical Methods, 2014 (15)

Capillary Electrophoresis (2.2.47)* (1)

Electrophoresis

- Micellar Electrokinetic Chromatography (MEKC)
- Capillary Isoelectric Focusing (iCEF)
- Capillary Gel Electrophoresis (CGE)
- Capillary Zone Electrophoresis (CZE)

- ❑ **GENERAL PRINCIPLES**
- ❑ **OPTIMISATION**
  - instrumental/electrolytic solution **parameters.**
- ❑ **SYSTEM SUITABILITY:**
  - retention time (only MEKC);
  - apparent no. of theoretical plates (*N*);
  - resolution or peak-to-valley ratio;
  - symmetry factor;
  - repeatability of area/(relative) migration time;
  - signal-to-noise ratio.
- ❑ **QUANTIFICATION**
  - **normalisation procedure** (exclude peaks due to solvents or any added reagents);
  - **relative peak areas** (corrected area(s) of peak(s)).

*This chapter has undergone pharmacopoeial harmonisation

Capillary Electrophoresis (2.2.47) (2)

The following monographs include reference to chapter 2.2.47:

Monograph	Type of technique
Aprotinin (0580)	CZE (purity)
Aprotinin concentrated solution (0[illegible]9)	CZE (Purity)
Erythropoietin concentrated solution (1316)	CZE (Identification)
Galantamine hydrobromide (2366)	CZE (Enanti[illegible]
Glutathione (1670)	[illegible] substances)
Human C1-esterase inhibitor (2818)	CZE (Production/molecular identification..)
Human alpha-1-proteinase inhibitor (2387)	CE (Production/isoform composition and protein structure)
Infliximab concentrated solution (2928)	**cIEF** (Production/charge variants)
Ropivacaine hydrochloride monohydrate (2335)	CZE (Enantiomeric purity)
Somatropin (0951), concentrated solution (0950), for injection (0952)	CZE (Charged variants)
Somatropin solution for injection (2370)	CZE (Deamidated forms)

chapter 2.2.47 becomes mandatory

Capillary Electrophoresis – Quiz

QUESTION: **Which parameters are allowed to be adjusted to some extent to satisfy the system suitability criteria without fundamentally modifying the methods for capillary electrophoresis technique?**

RESPONSE: There is nothing on adjustment of capillary electrophoresis conditions in the chapter 2.2.47. Instead, some possible adjustments might be sometimes given directly in monographs. For example, in *Somatropin* monographs, effective length of a capillary is given as at least 70 cm, rinsing times may be adopted according to the length of the capillary and equipment used, and injection time and pressure may be adapted in order to meet the system suitability criteria.

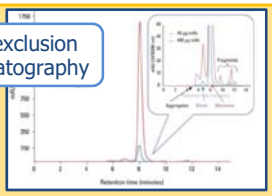
Capillary Electrophoresis (2.2.47)

- Provides a framework within which more detailed general chapters (*e.g.* methodologies applied to specific classes/families) can be written.
- Complements information in *general chapter on capillary isoelectric focusing for monoclonal antibodies currently under development*.

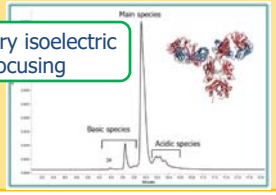
General Chapters for mAbs under Elaboration

- **General methodologies ('horizontal standards')**

Size-exclusion chromatography



Capillary isoelectric focusing



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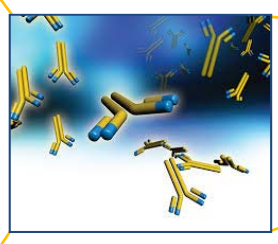
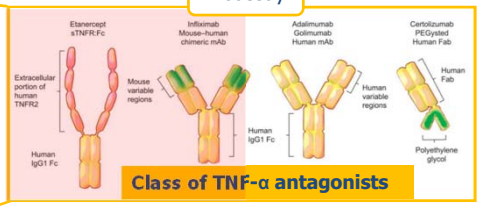


Bioassay



Class of TNF-α antagonists

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