New Frontiers in the Quality of Medicines

Workshop
Quality Standards for Biologicals

Moderators:
Mr Peter Castle
Dr Peter Jongen

EDQM International Conference
13-15 June 2007
Strasbourg, France
Methods, and difficulties encountered, in the elaboration of a new method:

Glycan Analysis

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*NIBSC, UK*

Why are glycan analysis monographs needed?

- An increasing number of biologicals are glycoproteins
- Glycosylation can affect functional activity in many ways
  - Direct effect on biological activity (e.g., receptor binding, complement activation, ADCC effects, ligand binding etc.)
  - Effects on *in vivo* half life (clearance rate, stability, proteolysis)
  - Possible immunogenicity (Neu5Gc or Galβ1-3Gal epitope)
  - Possible effects on shelf life due to different stability
- Glycosylation varies due to cell line, culture conditions etc.
- Complexities associated with “biosimilar” glycoproteins
- Wide variety of analytical methodologies used, but may not give comparable results
Monograph development is complex because...

- Protein glycosylation is complex and heterogeneous
- Protein glycosylation is of three main types
  - N-linked glycans (most common, most important)
  - O-linked glycans (quite common)
  - GPI-anchors (no products yet)
- Analysis can be carried out at the level of:
  - The intact glycoprotein
  - Peptide-glycopeptide mixtures
  - Cleaved glycan chains (different methods for O- or N-linked chains)
    - Sialylated chains
    - Desialylated chains
- Glycan analysis is technically difficult
- Optimal methodology depends on the information required

### Overview of glycan analysis methods

<table>
<thead>
<tr>
<th>Analysis at intact glycoprotein level</th>
<th>Analysis at the level of cleaved glycans</th>
<th>Analysis at the glycopeptide level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline borohydride (O-linked chains)</td>
<td>Hydrazine (O- and N-linked chains)</td>
<td>Enzymatic methods (N-linked chains)</td>
</tr>
<tr>
<td>Enzymatic methods (N-linked chains)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>MALDI mass spectrometry</td>
<td>MALDI mass spectrometry</td>
</tr>
<tr>
<td>MALDI or ESI mass spectrometry</td>
<td>Isoelectric focusing for sialylation heterogeneity</td>
<td>Kg spectrometry for sialylation heterogeneity</td>
</tr>
<tr>
<td>Capillary electrophoresis for sialylation heterogeneity</td>
<td>Ion exchange chromatography for sialylation heterogeneity</td>
<td>Lectin array approaches</td>
</tr>
<tr>
<td>Lectin ELISA approaches</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPAEC (Dionex) chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGC chromatography</td>
<td></td>
<td></td>
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<tr>
<td>MALDI MS</td>
<td></td>
<td></td>
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<tr>
<td>Fluorescent labeling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exo-glycosidase treatment</td>
<td></td>
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</tr>
<tr>
<td>MALDI or ESI mass spectrometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoelectric focusing for sialylation heterogeneity</td>
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</tr>
<tr>
<td>Capillary electrophoresis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reversed phase HPLC or MALDI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coupled HPLC-ESI-MS-MS (on PGC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coupled CE-ESI-MS-MS (novel)</td>
<td></td>
<td></td>
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<tr>
<td>Gel electrophoresis (FACE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Glycan analysis methods – NIBSC choices

Glycoprotein

- Analysis at the level of cleaved glycans
- Enzymatic methods (N-linked chains)
- MALDI or ESI mass spectrometry
- Fluorophore labeling
- Isoelectric focusing for sialylation heterogeneity
- Capillary electrophoresis for sialylation heterogeneity
- Anion exchange chromatography
- Reversed phase HPLC or MALDI
- Mass spectrometry

Existing monographs - Products

- Erythropoietin concentrated solution 01/2006:1316
  - Use of capillary electrophoresis to monitor sialylation profile
  - Total sialic acid content by resorcinol assay
- Alteplase for injection 01/2005:1170 corrected
  - 2- or 3- glycan chain variants by SDS-PAGE
  - Total sialic acid by thiobarbituric acid assay
  - Total neutral sugar by phenol-sulphuric acid assay
- Recombinant human coagulation Factor VIII 01/2005:1643
  - Monitor neutral sugar and sialic content or oligosaccharide profile
- Monoclonal antibodies for human use 07/2007:2031
  - May include oligosaccharide mapping for lot release
  - Glycosylation analysed during product and cell line characterisation
Existing monographs - Methods

- Capillary electrophoresis 01/2005:20247
  - No mention of application to monitoring sialylation profile
- Isoelectric focussing 01/2005:20254
  - No mention of use to show sialylation profile
- Mass spectrometry 01/2005:20243
  - No comment on use to monitor glycosylation heterogeneity in small glycoproteins – quantitation, resolution required etc.
- Peptide mapping 01/2005:20255
  - No mention of glycopeptides derived from glycoproteins
  - Glycosylation analysis will require coupled HPLC-mass spectrometry to analyse peptide/glycopeptide mixtures

Proposal 1: an introductory chapter

- An introductory chapter providing guidance on
  - When glycan analysis is necessary
    - The manufacturer will need an understanding of how glycosylation affects product quality
  - The different approaches which can be used
    - Intact glycoprotein
    - Cleaved glycan chains
    - Other methods
  - What information each of these approaches can provide
  - When it is appropriate to use each of these approaches
    - Defined in terms of the information needed for different product types
  - Which methodologies are currently described (at some level) in the EP
- This has been drafted for publication in Pharmeuropa
Glycan analysis of intact glycoproteins

- Mass spectrometry
  - Small proteins with relatively simple glycosylation
  - Define a fingerprint or seek accurate quantitation?
- Capillary electrophoresis or isoelectric focussing
  - Gives indication of sialylation profile
  - Crucial when sialylation defines the in vivo product half life
- Total neutral sugar or total sialic acid
  - Tells you it is a glycoprotein, and monitors consistency
- Monosaccharide analysis
  - Are methods sufficiently precise for useful information?

➢ Revise existing monographs to mention glycoproteins?

European Pharmacopoeia – rhEPO glycan analysis

- Biological activity of EPO largely controlled by in vivo half life
- This in turn depends on profile of sialylation
- Two methods assessed – IEF and capillary electrophoresis
- CE method in the monograph – this has legal status as the primary test method
- “Z-score” approach also used – as secondary methods
Glycoform analysis by ESI mass spectrometry

<table>
<thead>
<tr>
<th>Peak</th>
<th>Molecular weight (daltons)</th>
<th>Glycan chains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89865</td>
<td>1 O-glycan, 2 Neu5Ac</td>
</tr>
<tr>
<td>2</td>
<td>90523</td>
<td>2 O-glycans, 3 Neu5Ac</td>
</tr>
<tr>
<td>3</td>
<td>90811</td>
<td>2 O-glycans, 4 Neu5Ac</td>
</tr>
<tr>
<td>4</td>
<td>91470</td>
<td>3 O-glycans, 5 Neu5Ac</td>
</tr>
<tr>
<td>5</td>
<td>91763</td>
<td>3 O-glycans, 6 Neu5Ac</td>
</tr>
<tr>
<td>6</td>
<td>93421</td>
<td>4 O-glycans, 6 Neu5Ac</td>
</tr>
<tr>
<td>7</td>
<td>93162</td>
<td>1 biantennary fucosylated N-glycan and 2 O-glycans with 4 Neu5Ac</td>
</tr>
</tbody>
</table>

Assignments from molecular weight – no other direct evidence of glycosylation status

Coupled HPLC-MS/MS of (glyco)peptide maps

- Extension of peptide mapping to cope with glycosylation
- Provides information on site-specific glycosylation
- Probably need MS or MS/MS detection to be useful
- Glycopeptides tend to have poor sensitivity in MS
- Develop a fingerprinting approach
  - Not currently used for any products, as far as I am aware
  - Hard to set specifications for multivariate data sets
- Results could be strongly instrument dependent

- Not of immediate priority
- Reconsider when rewriting the peptide mapping monograph?
Priority - Profiling of cleaved oligosaccharides

- Widely used in product characterisation
- Different approaches used for N- and O-linked glycans
  - Enzymatic or chemical cleavage
- Several separation approaches used – examples include
  - “Dionex” high performance anion exchange chromatography
  - Fluorophore labelling and reverse- or normal-phase chromatography
  - Separate by charge (sialylation) and then underlying glycan structure
- Several approaches to define structures of components
  - Use of reference compounds
  - Sequential selective exoglycosidase digestion
  - Mass spectrometry
- For lot release assays, comparing profile probably sufficient

Glycan mapping of rhEPO: fluorescent labelling

Separation of differently sialylated glycan chains on a weak anion exchange resin. Fractions collected.

Separation of DEAE fractions on an amide-80 HPLC column, which is sensitive to the underlying glycan structure.

Yuen et al., *Brit. J. Haematol.*, 2003, **121**, 511-526
First steps – who does what and how?

• What methodologies are used by industry?
  – Industry survey, working with NPL in UK (11 labs)
  – Wider survey with USP (>100 responses)
• Initial emphasis on analysis of cleaved N-linked glycans
• How do the results from different analytical methodologies compare?
  – Produce materials for round-robin tests (glycan mixtures)
  – Working with NPL in the UK – funding from DTI M/B programme
  – Working with USP in the US and worldwide
  – Results should become available after September 2007
• Analyse the results for accuracy, precision, resolution
  ➢ Inform the drafting of method-specific monographs

Cleaved N-glycans: separation of neutral glycans

- HPAEC 27%
- HILIC 13%
- CE 21%
- RP-HPLC 7%
- NP 12%
- FACE 3%
- gel permeation 7%
- other 10%
Glycoproteins - inter-laboratory comparability

Glycan sample A contains mono-, di-, tri and tetra-sialylated chains. The chart shows variability in results from different labs for the amounts of each type of chain.

Proposal 2: Methodological monographs

- Choose a small number of widely used, “semi-generic” methodologies and draft more specific monographs, eg.
  - High performance anion exchange chromatography
  - Fluorophore labelling and HPLC separations
  - Weak anion exchange chromatography for sialylation/charge profile
- Leave flexibility in the monographs
  - Use of different anion exchange columns and detection systems
  - Use of different fluorophore labels
  - Use of different chromatographic approaches (depends on fluorophore)
- Mass spectrometric analysis of cleaved glycans?
- Provide advice on method – precision, accuracy, applicability?
Next steps – future monographs

- Need to investigate glycan cleavage reactions in future
  - Retain starting material used to prepare glycan reference mixtures
  - Circulate to labs for cleavage and glycan analysis
  - See how results differ from those previously provided
- Need to investigate O-linked glycan chains in future
- Consider other types of glycosylation – GPI anchors?
- Methodology to limit unwanted glycosylation?
  - Immunogenic substructures such as Gal\(\alpha\)1-3Gal, Neu5Gc
  - Glycan structures associated with plant or insect glycoproteins
- Keep “glycoproteins” separate from “synthetic glycoconjugates” such as glycoconjugate vaccines?

Working with the USP and others

- USP are also drafting monographs on glycan analysis
- Recognition that it is important that EP and USP monographs are consistent
- Exchange of ideas and draft monographs
- Mutual involvement in committees to harmonise views
- Topic has been proposed for formal harmonisation under the Pharmacopoeial Discussion Group process (May 2007)
- USP need for procedural reference materials
  - Method qualification
  - Instrument qualification
Proposed USP monograph structure
Four glycoprotein-orientated monographs proposed, to cover general approaches and more detailed methodology

- **<1084>** Glycoprotein and Glycan Analysis - Introduction and Choice of Analysis Methods
- **<1085>** Glycoprotein and Glycan Analysis – Deglycosylation of Glycoproteins
- **<1094>** Glycoprotein and Glycan Analysis – Monosaccharide Analysis
- **<1095>** Glycoprotein and Glycan Analysis – Oligosaccharide Analysis

Comparing methods – USP/NIBSC study

- USP and NIBSC are preparing N-glycan mixtures to be used in round-robin studies to assess method performance
- We are seeking information on the ability of different approaches to
  - Resolve individual glycan components
  - Identify as many components as possible
  - To quantify the components that are present
- Later, the parent glycoproteins will be used in another study to compare methods to cleave glycans from the peptide backbone
- Another study on O-glycan profiling may take place in the future
Acknowledgements

• NIBSC - C.-T. Yuen, Adrian Bristow
• USP - Tina Morris, Mike Ambrose
• EP Group 6 - Martin Schiestl (Sandoz) and USP BBPP Expert Committee
• USP ad hoc Group - Joe Siemiatkoski (Biogen IDEC)
• NPL - Smita Thobhani, Marc Bailey
QUALITY STANDARDS FOR BIOLOGICALS

The elaboration of monographs

Case study: Chondroitin sulphate sodium

Chondroitin sulphate sodium

1. The substance
2. The project
3. The monograph
4. The reference substances
5. Conclusion
1. The substance

What is it?

- Sulphated glycosaminoglycan
- Natural copolymer
- Disaccharide unit:
  - N-Ac-galactosamine & glucuronic acid

\[
\begin{align*}
\text{CS A} & \quad R = \text{SO}_3\text{Na} \text{ and } R' = \text{H} \\
\text{CS C} & \quad R = \text{H} \text{ and } R' = \text{SO}_3\text{Na}
\end{align*}
\]
Sources

Animal cartilage

- **Terrestrial (CS A):** cattle, pigs, chicken
- **Marine (CS C):** mainly sharks

Minor impact on specifications

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Use

- Borderline product: dietary supplement / medicinal product
- Pain relief in osteoarthritis (adjunctive therapy)
- Oral administration
2. The project

The project in figures

- 1 monograph
- 2 origins
- 5 group meetings
- 6 months of public enquiry
- 8 manufacturers involved
Step 1: Commission’s decision

- March 2001
- Addition to the work programme
- Referred to Group 6

*The European Pharmacopoeia work programme is widely distributed and available on our website*
Step 2: Towards a first draft

- 2001
  - September:
    - appointment of rapporteurs;
    - discussion of first manufacturer draft
  - October:
    - teleconference with 2 manufacturers
- 2002
  - March: discussion of revised draft

Step 2: Towards a first draft (cont’d)

- 2002
  - Summer: involvement of a 3rd manufacturer
  - September: draft finalisation
- 2003
  - April: Pharmeuropa 15.2
    - Public consultation
  - CRS establishment
Step 3: Taking feedback into account

- 2004
  - *March*: joint meeting with interested manufacturers
    8 companies, 6 countries
    Replacement of the method for related substances
    (small collaborative study)
  - *June*: submission to Commission
    ➔ Group asked to review assay limits

Step 4: Finalisation

- 2004
  - *September*:
    • Survey: limits approved for marketed products
    • Confirmation of suitability of the new test method

- 2005
  - *June*: resubmission to Commission + CRSs
    ➔ Adoption

- 2006
  - *January*: publication in 5.5*
  - *1 July*: implementation
* latest version in 6.0
3. The monograph

*Chondroitin sulphate sodium (2064)*

---

**Structure**

- **Definition**
  - Description (main components)
  - Origins
  - Content limits
- **Characters**
  - Appearance & solubility
    - Non mandatory
- **Production**
  - Control of starting materials (animals)
Structure (cont’d)

- Identification
  - IR*
  - Na reaction
  - Electropherograms in the test for related substances

- Tests
  - pH
  - Specific optical rotation*
  - Intrinsic viscosity

* discriminates according to origin: marine/terrestrial

Structure (cont’d)

- Tests (cont’d)
  - Related substances
    Agarose gel electrophoresis
    Global limit: 2 per cent

- Dermatan sulphate
- Sodium hyaluronate
- Heparan sulphate
- Keratan sulphate
Structure (cont’d)

- Tests (cont’d)
  - Protein
  - Chlorides
  - Heavy metals
  - Loss on drying
  - Microbial contamination

Structure (cont’d)

- Assay
  - Cetylpyridinium chloride titration
  - Content assigned on the basis of sulphonate determination
- Storage
  - Non mandatory
- Labelling
  - Only information that allow to demonstrate compliance
4. The reference substances

*Chondroitin sulphate sodium CRS*

*Chondroitin sulphate sodium (marine) CRS*

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Reference substances

- *Chondroitin sulphate sodium CRS*
  - Bovine origin
  - Candidate checked against manufacturers’ in-house standards
    - harmonisation
  - To be used whatever the origin

- *Chondroitin sulphate sodium (marine) CRS*
  - To confirm identification (IR)
5. Conclusion

Key ideas

- Monograph elaboration is an open process
- Check our work programme
- Get involved early
- Just a part of Group 6’s work
  (also synthetic peptides, rDNA proteins)
Thank you for your attention!
Background 1: the drug

GCSF is a 19kD cytokine, which acts on granulocyte precursors to produce mature neutrophils.

It is naturally O-glycosylated, but the non-glycosylated form is biologically active.

Used to accelerate recovery of neutrophil counts following chemotherapy.
Background 2: the environment

<table>
<thead>
<tr>
<th>Non-glycoslated GCSF</th>
<th>INN Filgrastim</th>
<th>Product Neupogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylated GCSF</td>
<td>INN Lenograstim</td>
<td>Product Granocyte</td>
</tr>
</tbody>
</table>

GCSF placed on the work program of group 6 1996. Suspended 3 years later due to lack of progress.

Patent expiry (filgrastim) 2006

Work recommenced 2004/2005

Key decision: Filgrastim and Lenograstim are separate substances, and will require two different monographs. Elaboration of the two can proceed separately. Filgrastim has reached Pharmeuropa stage. Lenograstim is stalled.

### Background 2: the environment (2007)

<table>
<thead>
<tr>
<th>Drug substance</th>
<th>Manufacturers</th>
<th>Specifications submitted</th>
<th>Comments on the draft</th>
<th>Reference Material offered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filgrastim</td>
<td>One Innovator</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Three Biosimilars (not yet licensed)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lenograstim</td>
<td>One innovator</td>
<td>No</td>
<td>No draft</td>
<td>No</td>
</tr>
</tbody>
</table>

© NIBSC
The Filgrastim draft monograph.

Current status: Published in Pharmeuropa. Comments to be discussed At group 6 in September

PA/PH/Exp./ T 098 17 ANP

---

FILGRASTIM CONCENTRATED SOLUTION
Filgrastimi solutio concentrata

**DEFINITION**

16. Solution of a protein having the structure of the granulocyte colony-stimulating factor
17. produced and secreted by certain human blood cell types. The protein stimulates the
18. differentiation and proliferation of leukocyte stem cells into mature granulocytes.
19. 
20. Content: minimum 1.5 mg of protein per milliliter.
21. Purity: minimum 1.0 x 10^6 EU per milligram of protein.

---

Draft monograph:

Based on

1. Input from manufacturers

2. EUROPEAN PHARMACOPOEIA COMMISSION
   PA/PH/Exp./ T (98) 55
   Technical Guide for the elaboration of
   Monographs for Biologicals
Filgrastim concentrated solution:
Draft monograph structure

Drug structure
Definition
Production statement
Characters
Identification
Tests
Bacterial endotoxin
Assay
Labelling

Filgrastim concentrated solution:
Draft monograph structure

Drug structure
Primary structure defined
Definition
Production statement
Characters
Identification
Tests
Bacterial endotoxin
Assay
Labelling

NIBSC
Filgrastim concentrated solution:
Draft monograph structure

Drug structure
Definition
Production statement
Characters
Identification
Tests
Bacterial endotoxin
Assay
Labelling

Defines the drug, and states:

- minimum concentration (1.5mg/ml)
- minimum potency (1.0 x 10^8 IU/mg protein)

States production by recDNA technology, and that host cell protein and DNA limits will apply.
Filgrastim concentrated solution:
Draft monograph structure

Drug structure
Definition
Production statement
Characters States what it looks like
Identification
Tests
Bacterial endotoxin
Assay
Labelling

A Cross-reference to the biological assay
B Cross-reference to IEF (iso-electric focusing)
C Cross reference to HP-SEC (test for high mol wt impurities)
D Cross reference to SDS PAGE (reducing and non-reducing)
E Peptide mapping
F N-terminal sequence analysis
Filgrastim concentrated solution:
Draft monograph structure

Drug structure

Definition

Production statement

Characters

Identification

Tests

Bacterial endotoxin

Assay

Impurities with higher molecular masses (HP-SEC). <2.5%

Impurities with different molecular masses (SDS-PAGE) No band > 2%

Impurities with different charges (IEF) No band > 20%

Related Proteins (RP-HPLC) <2% (individual impurities), <3% (total)

Labelling

<2IU/mg protein
Filgrastim concentrated solution:
Draft monograph structure

Drug structure
Definition
Production statement

Characters
Identification
Tests
Bacterial endotoxin
Assay
Labelling

Labelling
States Content and potency

a) Protein – determined from test for related proteins and proposed CRS with a defined content, and
b) Biological activity – determined by proliferation of NFS-60 cells, in IU
80-125% of stated potency (fiducial limits 74-136%)
Issues:

1 Scientific
2 Non-scientific
- The draft monograph, and the specifications from which it is derived, are very traditional. There is no MS, no CZE, no LC-MS etc etc.

- The reference material. The monograph is heavily dependent on a putative CRS. It may not be possible, for technical reasons, to prepare and distribute this for GCSF. Can we consider a RM-independent monograph?

- Can we consider a RM-independent monograph?

Peptide map
Profiles obtained with test and reference solutions correspond
Analysis of tryptic digest of interferon alpha-2 using MALDI-TOF MS peptide profiling and database searching

Jun Wheeler, NIBSC

After digestion, digest was desalted using ZipTip C18. The samples with the matrix (alpha-cyano-4-hydroxycinnamic acid, 10 mg/mL in 50% acetonitrile 0.1% TFA) were applied onto the target. MALDI mass spectra were recorded with Autoflex MALDITOF operated in the positive ion reflection mode at 20 kV accelerating voltage with pulsed ion extraction enabled.

Mass spectrum - peptide mass profile from interferon digest
Mass list

[Images of mass spectrum and mass list]

Propen View
Start to
Stop: Observed
Score: 136
Score: Confidence
Delta: 0.000 
Hit Sequence

[Table of peptide mass data]
Issues:

1 Scientific
2 Non-scientific

Filgrastim is unusual. Previous Biotech monographs have been drafted in an environment where there is no patent position (e.g. GH, insulin), or where the innovators have collaborated before biosimilars exist (e.g. EPO).

The current environment, where we have licensed innovator product at end of patent life, and unlicensed bio-similars, but with well-developed products and specifications, may be the first of what is the more usual situation.

The situation raises a number of specific questions
- Do the methods and specifications of the unlicensed bio-similar products have any standing when it comes to drafting the monograph?
- If not, can the innovator effectively terminate the project by declining to submit a specification?
- Should the relationship between the EP monograph and bio-similar status be more clearly defined?
- The various specifications are broadly similar but differ in detail. What are the implications of adopting a monograph which would require the manufacturer to abandon several years of development work?
- How do we answer the question “What purpose does the monograph serve in this environment?”
- Can we proceed in the absence of a RM?

On behalf of Group 6, thank you for your attention

Groupe d’experts/Group of Experts No 6
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Biological Substances

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