Need for more accurate anti-A / B and anti-complementary assay methods for human intravenous immunoglobulin release

C. de COUPADE
Cell Biology Lab, LFB BT
PPB 2013 - Lanzarote
Monograph for Human normal immunoglobulin for intravenous administration

- 01/2012: 0918

Required tests:

- Solubility
- pH
- Osmolality
- Total protein
- Protein composition
- Molecular size distribution
- Anticomplementary activity
- Anti-A and anti-B haemagglutinins
- Anti-D antibodies
- Antibody to hepatitis B surface antigen
- Immunoglobulin A
- Water
- Sterility
- Pyrogens

Mastering critical steps in the process improves clinical safety for the patients
Monograph for Human normal immunoglobulin for intravenous administration

01/2012: 0918

Required tests:

- Solubility
- pH
- Osmolality
- Total protein
- Protein composition
- Molecular size distribution
- **Anticomplementary activity**
- **Anti-A and anti-B haemagglutinins**
  - Anti-D antibodies
  - Antibody to hepatitis B surface antigen
  - Immunoglobulin A
- Water
- Sterility
- Pyrogens
The anti-A and anti-B isoagglutinin levels measurement
Incident of severe haemolysis following IVIG therapy

→ Limit the levels of anti-A and anti-B antibodies

Determination of anti-A/B using the Eur. Pharmacopeia assay (2.6.20)

- Indirect method using the human antiglobulin
- Direct method: No agglutination should be observed when IVIG (25 g/L) is diluted more than 64-fold

→ 2009: Modification of the monographs of *Human normal immunoglobulin* (0338) and of *Human normal immunoglobulin for intavenous administration* (0918)
Objective: To validate the direct « spin » haemagglutination method using papain-treated erythrocytes [Thorpe SJ et al., “International collaborative study to evaluate candidate reference to standardize haemagglutination testing for anti-A and anti-B in normal intravenous immunoglobulin products”; Vox Sanguinis 97, 160-168 (2009)]

Positive control 07/306 (5% IVIG)
Negative control 07/308 (from blood group AB only)
Limit reference preparation 07/310 (5% IVIG with nominal anti-A/B titers of 64)

→ A two-fold difference not considered significant → overall up to 16-fold variations in titers
A new cytometry method applied to anti-A and anti-B level determination

Principle of measurement of anti-A/B by flow cytometry

→ Linear dose-response function
→ Results are expressed as the ratio between the sample line slope and the positive control IVIG standard line slope
BM5  to anti-a and anti-b level determination?
BEAUDET Marie; 2013-05-02

BM6  RBC, HQC, LQC : abréviations à expliquer à l'oral,
BEAUDET Marie; 2013-05-02
Two quality controls (QC) were used during anti-A and anti-B assays (High and Low)

Results expressed as slope ratio between high and low QCs

→ High inter-assay reproducibility of the flow cytometry method
Three different batches tested for each product

**A- Direct haemagglutination**

<table>
<thead>
<tr>
<th>IGIV preparation</th>
<th>Anti-A</th>
<th>Anti-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClairYg</td>
<td>4-8-4</td>
<td>8-8-8</td>
</tr>
<tr>
<td>IgIV1</td>
<td>4-8-4</td>
<td>4-16-4</td>
</tr>
<tr>
<td>IgIV2</td>
<td>8-8-8</td>
<td>16-16-16</td>
</tr>
<tr>
<td>IgIV3</td>
<td>16-16-8</td>
<td>32-16-16</td>
</tr>
<tr>
<td>IgIV4</td>
<td>8-8-8</td>
<td>16-16-16</td>
</tr>
</tbody>
</table>

**B- Flow cytometry**

- High precision with the flow cytometry method
- May allow fine process optimization monitoring
Specific lysis of human red blood cells (hRBC) from A, B and O RhD negative induced by 5 liquid IVIG products in the presence of guinea-pig complement.

→ Good correlation between anti-A/B levels and physiological effect (haemolysis of RBC)
The flow cytometry method presents

- Increased reproducibility
- Increased robustness
- Increased accuracy

... compared to the direct haemagglutination method

→ Revision of the current Eur. Pharmacopeia section 2.6.20?
→ Would fractionators agree on replacing the existing historical method?
The anti-complementary activity (ACA) test
Regulatory requirements for IVIG drug release

Presence of antibody aggregates in a therapeutic product can affect drug efficacy and even cause immunogenic reactions when administered to patients.

Need for accurate measurement of aggregation content $\rightarrow$ SEC

Functional aggregation content

Immune complex-like aggregates $\rightarrow$ ACA

Others impurities complement activators
Anticomplementary activity test
Monograph 2.6.17 of the European Pharmacopeia 6.6

Complement activation and consumption

Human immunoglobulin concentrate + Guinea-pig complement

Residual amount of complement

Residual complement titration

Sensitized sheep RBC (loaded with rabbit antibodies against sheep RBC, haemolysin)

Residual complement to be titrated

Lysis

The ACA is expressed as the % of consumption of complement relative to the complement control, which is considered as 100%

Specifications for IVIG ≤ 50%
The anti complementary assay (ACA)

Where are we today?
EDQM collaborative study to establish human immunoglobulin reference preparation

- 8 laboratories (nb in box represent the lab codes)
- ACA according to Eur. Pharm.
- In-house/commercial complement preparation
- IVIGs formulated at pH 7

- Large distribution of ACA activity among laboratories
- ACA activity is usually either high (lab 12), medium (lab 11) or low (lab 15)

EDQM collaborative study to establish human BRP (Sandberg E et al, “Calibration of the human immunoglobulin BRPs for ACA and molecular size (batch 1) and for Fc function and molecular size (batches 1 & 2)”; Pharmeuropa Bio&SN98 (2012))
Identified critical parameters for ACA assays

Influence of complement batch


→ Each Lab can select the complement batch to be compliant with the acceptance criteria of the ACA Eur. Pharm. method
Identified critical parameters for ACA assays

Sample preparation

EUROPEAN PHARMACOPOEIA 5.0

2.6.17. Test for anticomplementary activity of immunoglobulin

<table>
<thead>
<tr>
<th></th>
<th>Condition A</th>
<th>Condition B</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH of buffer solution</td>
<td>7.3</td>
<td>8.9</td>
</tr>
<tr>
<td>pH of complement control</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>pH of sample</td>
<td>6.4</td>
<td>7.3</td>
</tr>
<tr>
<td>ACA Lot A (%)</td>
<td>45</td>
<td>34 (-11)</td>
</tr>
<tr>
<td>ACA Lot B (%)</td>
<td>38</td>
<td>27 (-11)</td>
</tr>
<tr>
<td>ACA Lot C (%)</td>
<td>46</td>
<td>38 (-8)</td>
</tr>
</tbody>
</table>

→ Strong impact of pH adjustment on ACA results
→ Any standardization of the pH adjustment method?
High ACA activity and patient adverse reactions

No correlation between high ACA value and safety

ABSTRACT (*)

Quality assurance release levels for anticomplementary (AC) activity of a non-modified intravenous immunoglobulin (IGIV, pH 4.25) were reviewed over a period of one year and nine months in an attempt to correlate the incidence of complement-mediated-type adverse reactions with the AC level. Over 200 lots and ten possible complement-mediated side effects were evaluated. No correlation was found. A similar evaluation was made of a prospective study in which three lots of IGIV, pH 4.25 (one with a low, one with a mid-range, and one with a high AC activity) were used; again no correlation was found. Finally, a comparison was

The ACA assay: general feedbacks

Questions still remain around the ACA method

- Complexity of reagents and high impact of complement lot
- High inter- and intra-laboratory variability
- No or weak clinical relevance
- Historical method → sample preparation not optimized for acidic formulated IVIGs

What could be the alternatives?

1. Suppress the ACA test
2. Adapt the ACA test to low pH formulated Igs
3. Replace ACA test with alternative methods
Alternative tests to ACA to measure anticomplementary power:

ELISA for C1q binding
&
for C5a release
Complement activation pathways

- Classical Pathway:
  - Ag-Ab complexes and others (e.g. CRP)
  - C1q, C1r, C1s
  - C1-INH

- Lectin Pathway:
  - Microbial surfaces (mannose) and others (e.g. IgA)
  - MBL, MASP

- Alternative Pathway:
  - Spontaneous and foreign surfaces, e.g. LPS
  - C3(H2O)

 Released C5a ELISA

C1q ELISA
How relevant is C1q binding measurement?

- C1q binding used as an *in-vitro* dosage of circulating immune complexes able to bind the C1q in human serum (autoimmune diseases)

- Assay for the detection and quantitation of C1q binding aggregates (Georgakopoulos T et al, “C1q aggregate binding for the determination of anti-complementary activity of immunoglobulin products”; Biologicals 39 (2011) 38-42)

- Correlation between C1q binding and ACA for aggregates generated at neutral pH

- C1q binding assay highly sensitive to Igs structural modification
## Fidelity & Linearity study

### C1q

<table>
<thead>
<tr>
<th>Run</th>
<th>Operator 1 Day 1</th>
<th>Operator 2 Day 1</th>
<th>Operator 3 Day 2</th>
<th>Operator 1 Day 2</th>
<th>Operator 2 Day 2</th>
<th>Operator 3 Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.01</td>
<td>1.02</td>
<td>1.06</td>
<td>1.00</td>
<td>1.05</td>
<td>1.03</td>
</tr>
<tr>
<td>2</td>
<td>1.05</td>
<td>1.02</td>
<td>1.00</td>
<td>1.00</td>
<td>1.03</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>0.96</td>
<td>1.02</td>
<td>1.00</td>
<td>1.00</td>
<td>1.02</td>
</tr>
<tr>
<td>4</td>
<td>0.94</td>
<td>1.02</td>
<td>0.99</td>
<td>1.05</td>
<td>1.09</td>
<td>1.02</td>
</tr>
<tr>
<td>5</td>
<td>1.06</td>
<td>1.00</td>
<td>1.00</td>
<td>1.10</td>
<td>1.10</td>
<td>1.02</td>
</tr>
<tr>
<td>7</td>
<td>1.04</td>
<td>1.01</td>
<td>0.96</td>
<td>1.04</td>
<td>1.02</td>
<td>0.91</td>
</tr>
<tr>
<td>8</td>
<td>0.99</td>
<td>1.07</td>
<td>1.07</td>
<td>1.00</td>
<td>1.04</td>
<td>1.19</td>
</tr>
</tbody>
</table>

**CV_R = 8% (k=1 et n=1)**

**Linearity**

![](chart.png)

### C5a

<table>
<thead>
<tr>
<th>Run</th>
<th>Operator 1 Day 1 Pool S 1</th>
<th>Operator 2 Day 2 Pool S 1</th>
<th>Operator 1 Day 2 Pool S 1</th>
<th>Operator 2 Day 2 Pool S 1</th>
<th>Operator 1 Day 3 Pool S 1</th>
<th>Operator 2 Day 2 Pool S 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>0.9</td>
<td>0.9</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>0.9</td>
<td>0.9</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**CV_R = 9.8% (K=1 et n= 1)**

**Response window between 0.4 and 2.5**

- Good precision for both methods
- Linearity observed for C1q assay
Methods and performance

- Sensibility to complement activators: (1/2)
  - Ig aggregation induced by heating (pH 7.0; 63°C for 5, 10 or 15 min)

→ Good correlation between generated polymers and C1q binding/C5a release
Methods and performance

- Sensibility to complement activators for C1q assay (2/2)

<table>
<thead>
<tr>
<th>Ig aggregation induced by stirring</th>
<th>Presence of immune complexes) (LPS or tetanus toxoid)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

- Increased C1q binding in acceptable limits (~1)
- Increased C1q binding with presence of immune complexes (>1)
Limits of alternative methods

- Determination of a threshold value for C1q & C5a correlated with immunologic reactions resulting from non specific complement activation: A challenge

- Relevant quality controls? No batch available with known adverse effect in human

- No existing animal model with direct links between complement activation and physiological effect → to be developed?
Open discussion on the ACA test

- Weaknesses of the ACA test:
  - Complex reagents and strong impact of the complement batch
  - High intra-inter laboratory variability
  - Physiological relevance
  - Sample preparation (adaptation to acidic Ig)

⇒ Suppress the ACA test?
⇒ Adapt the ACA test to low pH formulated Ig?
⇒ Replace ACA test with alternative methods?
  (C1q binding and/or C5a release)
Some of the Eur. Pharmacopeia methods may need to evolve towards more accurate and standardized assays

Some of the Eur. Pharmacopeia methods are usually not compliant with the Q2R1 validation guidelines

Due to complex method of production and various processes, different IVIG may exhibit meaningful differences in terms of safety

→ Why not applying the most predictive and reproducible \textit{in vitro} test to control IVIG products?
Special thanks to...

- The Cell Biology Lab; LFB BT (B. Bodenant, A. Pottin, C. Souvannavong)
- Frédéric DHAINAUT: Head of Pharmacology and Bioanalysis Department; LFB BT
- Paul MARTRES: Director of Non Clinical Department; LFB BT
- Gérald PERRET: Biomolecular Characterisation Lab Manager; LFB BT
- Philippe PAOLANTONACCI: Head of Process Unit; LFB BT
- Ludovic BURLOT: Director of Biopharmaceutical Department; LFB BT