Department of Physics, Chemistry and Biology

Master's Thesis

Investigation of a Method for Determination of Anticomplementary Activity (ACA) in Octagam®

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QC Methodology

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Abstract

This Master Thesis was conducted at Octapharma AB in Stockholm.

Anticomplementary activity (ACA) is a measure of the product’s abilities to activate the complement system. IgG aggregates are mainly responsible for this activation. Two different performances of a method for determination of ACA in Octagam® are available. The two performances are based on the reference method for test of ACA in immunoglobulins in the European Pharmacopoeia Commission Guideline 6.0 (chapter 2.6.17). The method is carried out either in test tubes or on microtiter plates. The test tube method can be performed either in a manual manner or modified, being more automated. The latter performance has been applied in this study. The plate method is more automated than both of the tube methods. The plate method and the manual tube method have earlier seemed to result in different outcomes, which was the basis for this thesis.

The plate method and the modified test tube method have been compared and robustness parameters have been studied in order to see which factors influence on the end result. The adequacy of using Human Biological Reference Preparation (human BRP) as a control for the ACA method in general has also been investigated. Samples of the product are outside the scope of this thesis and have not been investigated.

According to this study, the plate method and the modified tube method are not comparable with regard to complement titration results and to ACA of the BRP control. A higher precision is gained with the plate method. This in combination with the higher degree of automation makes the plate method advantageous in several aspects. When it comes to the robustness of the ACA method in general, the sheep red blood cells (SRBC) used are critical. Haemolysin dilution and complement activity seem to be critical as well.

Human BRP is, according to this study more adequate as a reference for the plate method than for the tube method. An In house control is believed to be more representative to the ACA method in general as it is of the same nature as the samples analysed, in contrast to the human BRP.
Sammanfattning

Det här examensarbetet utfördes på Octapharma AB i Stockholm.


Plattmetoden och den modifierade rörmetoden har jämförts och robusthetsparametrar har studerats för att se vilka faktorer som påverkar slutresultatet. Frågan om det är lämpligt att använda Human Biological Reference Preparation (human BRP) som kontroll för ACA-metoden har även undersökts. Produktprover har inte undersömts i examensarbetet.


Human BRP är enligt denna studie mer lämpad för plattmetoden än för rörmetoden. En In-house-kontroll antas vara mer representativ för ACA-metoden i allmänhet på grund av att den har samma egenskaper som de analyserade proverna till skillnad mot human BRP.
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### Acronyms

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<th>Definition</th>
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<tr>
<td>ACA</td>
<td>Anticomplementary activity</td>
</tr>
<tr>
<td>GBBS</td>
<td>Gelatine barbital buffer stock solution</td>
</tr>
<tr>
<td>RT</td>
<td>Room tempered</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells, -S sensitized with haemolysin</td>
</tr>
<tr>
<td>BRP pos. control</td>
<td>Human Biological Reference Preparation positive control (batch 3)</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IVIg</td>
<td>Intravenous immunoglobulin</td>
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</tbody>
</table>

### Keywords

Anticomplementary activity (ACA), BRP positive control, European Pharmacopoeia, plate performance, test tube performance
1 Introduction

Before releasing a batch of a plasma product to the market, it has to fulfil several quality requirements. Except from the highly controlled production process, one of the quality controls of the end product of Octagam® concerns its anticomplementary activity (ACA).

There are two different performances that can be applied for determination of ACA in Octagam®. They are both based on the test for anticomplementary activity in immunoglobulins, presented in section 2.6.17 in the European Pharmacopoeia Commission Guideline 6.0. The method is carried out either in test tubes with manual dilution, or more automated on microtiter plates.

Regarding the outcome of ACA, the two performances have seemed to differ. Workshops have been held in order to understand the mechanisms influencing on the ACA assay and to discuss possible technical simplifications and improvements that can be done to reduce the workload the assay entails.

As mentioned, the plate carryout is a more automated way of working with ACA compared to the manual tube method. Applying the manual tube method, all dilution series in the assay are performed manually on an ice bath. The plate method involves a robot pipetting machine for dilution steps. Robot handling of samples is both timesaving and enables a higher precision as the human erroneous aspect is not influencing on the pipetting. Also, it requires less work from the analyst. In addition to that, it is easier to handle a larger number of samples by automated systems than by hand.

Following one workshop, some changes in the plate performance – for ACA titrations as well as for the ACA assay, were proposed. Less automation was recommended as the performance described in the European Pharmacopoeia method is based on manual handling of samples. The new ACA performance to be implemented was the test tube method with some modifications.

The majority of the suggestions for the modified tube method are presented in table 1. Plate performance is presented as well.

Table 1. The table shows differences between the plate method and the modified tube method.

<table>
<thead>
<tr>
<th>Part of the assay</th>
<th>Plate method</th>
<th>Change suggestions (modified tube method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatine barbital buffer stock solution (GBBS)</td>
<td>GBBS is room tempered (RT) when used.</td>
<td>All GBBS that comes in touch with complement should be cooled in 2-8°C before use to avoid unwanted biological reactions.</td>
</tr>
<tr>
<td>Gelatine for the gelatine buffer</td>
<td>Supplier is Becton, Dickinson and Company, USA</td>
<td>Merck, USA was suggested as supplier, since differences in quality of different gelatine suppliers have been observed in the past.</td>
</tr>
<tr>
<td><strong>Sheep red blood cells (SRBS) and sensitized SRBC (SRBC-S)</strong></td>
<td>There are no restrictions concerning when during assay performance the suspensions should be prepared. RT storage is accepted for SRBC and up to 6 h of storage in 2-8°C is accepted for SRBC-S.</td>
<td>SRBC as well as SRBC-S should preferably be used directly after preparation, else they could be stored in 2-8°C up to 6 h.</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>First incubation</strong></td>
<td>Directly after 1 h incubation, samples and controls are diluted in an automatic robot with RT GBBS.</td>
<td>After one hour of incubation, the test tubes should be cooled in an ice bath for at least 5 min before dilution in an automatic robot with cold GBBS.</td>
</tr>
<tr>
<td><strong>Second incubation</strong></td>
<td>Post addition of SRBC-S to the dilution series, incubation mixtures are transferred from test tubes to a microtiter plate. Incubation is thereafter performed in a 37°C air-filled incubator for 1 h. After 1 h incubation, the plate is cooled in a 2-8°C refrigerator for at least 5 min. After cooling, the microtiter plates are directly centrifuged for 5 min. One centrifugation is required to take all samples.</td>
<td>Incubation of test tubes should be performed directly without any transfer, in a 37°C water bath for 1 h. After 1 h incubation, the test tubes should be cooled down in an ice bath for at least 5 min. After cooling, the test tubes should be transferred to racks to be centrifuged for 5 min. Two centrifugations are required to take all samples.</td>
</tr>
</tbody>
</table>

Regarding the suggestions for the modified tube method, the same gelatine as before (Becton, Dickinson and Company, USA) was still used for preparation of GBBS since the gelatine buffer was opalescent with the other gelatine (Merck, USA), regardless how the buffer was prepared. Apart from the gelatine issue, the rest of the change suggestions were adopted for the modified tube method.

Ever since the modified tube method was adopted, the Human Biological Reference Preparation positive control, batch 3 (BRP pos. control) has been low and seldom met its requirement, which has resulted in many non-approved assays of ACA. However, there are no problems getting the assays approved with the manual test tube method.
1.1 Aim

This diploma work aims to answer the following questions concerning the method for determination of anticomplementary activity (ACA) in Octagam®.

- Is the plate and the modified test tube method comparable?
- Which parameters in the method in general, influence on its robustness?
- Is the BRP control adequate as a reference for the method in general?
- Is the BRP positive control sensitive to different predilutions of the complement?

By putting raw data together and combine this with studies performed during the thesis, conclusions could be drawn.
2 Background

2.1 Human Plasma

Human plasma consists of hundreds of different proteins, many of them with unknown functions. Albumin and immunoglobulin G (IgG) together represent 80% of all plasma proteins, taking up 35 and 10 g/L respectively. Other proteins in the plasma are protease-inhibitors such as α1-antitrypsin, antithrombin and coagulation factors such as for example factor VIII.1

Today, 20 of the plasma proteins are used for treatment of bleeding and thrombotic disorders, immunological diseases, infectious conditions and tissue degenerating diseases. Thus, human plasma products are saving countless of patients from life-threatening conditions.1

Yearly, more than 28 million litres of human plasma are fractionated throughout the world.1

2.2 Immunoglobulin G (IgG)

Immunoglobulins are classified by their physical, chemical and immunological properties. IgG is the most common of the different classes of antibodies.2

The IgG molecule composes of four polypeptide chains, two light chains and two heavy chains. These together build up two antigen-binding units, where each unit consists of one light and one heavy chain. The IgG molecule is said to be bivalent because of that, able to bind two identical epitopes.2

The IgG molecule has both constant and variable regions. The constant regions are the same for all IgG molecules but the variable regions differ between each molecule, giving every IgG molecule different binding properties. Each antigen-binding site recognizes a certain antigen and each immune system consists of billions of different antigen-binding sites on antibodies. The diversity depends on random recombination and mutation of the genes involved in the building-up procedure of the variable regions.2

2.3 History of Intravenous Immunoglobulins

Immunoglobulins were for a long time only suitable for intramuscular administration. The intramuscular immunoglobulins could only be used as prophylaxis, to treat patients having different infections, for example hepatitis A. Patients who received intravenous doses were subjects to severe systemic reactions. These reactions were thought to arise due to Ig aggregates formed in the manufacturing procedure. The aggregates had the ability to activate complement resulting in the grave consequences. Therefore, patients suffering from primary and secondary deficiencies could for a long time not be treated properly.3

Not before the 1970s, fractionated immunoglobulins could be administrated intravenously. Intravenous immunoglobulins (IVIg) resulted in an increasing usage of immunoglobulins.
Immunoglobulins are today dominating the plasma market throughout the world, representing 39% of all plasma products.

### 2.3.1 IVIg Preparations and Immunodeficiency Diseases

IVIg preparations are used for treatment of patients suffering from different diseases affecting the immune system. They are used for treatment of autoimmune and systemic inflammatory diseases and their immunomodulatory effects are achieved by complex interactions between the pharmaceutical preparation and the immune response. Some of the actions of IVIg are blocking of Fc-receptor function, suppression of auto-reactive T-cells, modulation of complement activation, modulation of inflammatory mediator production (e.g. cytokine), and down-regulation of macrophage activity. Furthermore, IVIg preparations are thought to down-regulate antibody production by B-cells and to regulate the passage of auto-immune cells across the blood-nerve barrier.

IVIg preparations can be used to treat primary immunodeficiencies including X-linked agammaglobulinemia, hypogammaglobulinemia and common variable immunodeficiency (CVID). Later on, IVIg preparations have also been proved useful to treat secondary immunodeficiencies, preventing the patients from severe infections. Patients with secondary immunodeficiencies are not having innate defects of their immune response. Instead, for example treatment with immune suppressive agents has knocked out their defence against pathogens. Hence, secondary immunodeficiencies are deficiencies resulting from other diseases such as for example multiple myeloma and after bone marrow transplantations.

IVIg therapy has also turned out to be successful for treatment of children infected by HIV, by prolonging the infection-free time of the individual. Furthermore, IVIg treatment has been shown to be effective to patients with Guillain-Barré syndrome (GBS), chronic inflammatory demyelating polyneuropathy (CIPD) and to increase the platelet counts in children with idiopathic thrombocytopenic purpura (ITP).

When it comes to therapeutic applications of IVIg, neuromuscular diseases like GBS and CIPD have been extensively studied.

### 2.3.2 Octagam® 5%

Octapharma manufactures the human IVIg product Octagam® 5% which is the best-seller product of the company. Last years, the preparation has successfully been introduced to the USA.

Octagam® 5% contains 5% of human Ig, formulated in 10% maltose. It is a liquid preparation stable at room temperature and has a storage time of two years at any temperature in the interval 2-25°C.

The dominating antibody in IVIg preparations is IgG. More than 99.6% of the IgG molecules in Octagam® are structurally monomeric or dimeric. These structures are the ones associated with the therapeutic effect of the preparation. Less than 1% of the IgG molecules are polymeric, a structure associated with adverse reactions when administered in high doses.
### 2.4 Fractionation Technology

Fractionation is the industrial process in which therapeutic plasma proteins are isolated in different extracted fractions. Factories manufacturing plasma products are operated in compliance with Good Manufacturing Practice (GMP) and the production process is highly regulated by authorities.\(^1\)

Cohn and his co-workers developed the basics of the fractionation procedure of blood plasma used today in the 1940s. Cohn fractionation technology was initially designed to obtain albumin but has later on been developed for selective precipitation of several proteins using defined ethanol concentrations combined with shifts in pH, temperature and osmolality. Today, more than 20 different protein products can be extracted in large-scale processing of a plasma pool. The precipitates are extracted by centrifugation or filtration. The fractions extracted in the fractionation procedure are purified further into individual therapeutic products.\(^1\)

Last years, the complexity of the fractionation procedure has increased by introduction of several chromatographic methods. Chromatography techniques are now used to increase the purity of the products, for isolation of new proteins from the fractions, for extraction of trace labile proteins, for improvement of the protein recovery and for removal of viral inactivation agents.\(^1\)

Octagam® is prepared from plasma of a large donor pool and is manufactured by Cohn’s alcohol fractionation process.\(^12\)

Octagam® is treated with two distinct virus inactivation steps to assure viral safety. The solvent detergent (SD) method is one of them. In SD, lipid-enveloped viruses (e.g. HIV, hepatitis B and hepatitis C\(^9\)) are inactivated through destruction of the lipid coat and the binding site of the virus’ surface. SD treatment is utilising 0.3% tri(n-butyl)phosphate (TNBP) and 1% Triton X-100 in 6°C for 4 h for inactivation of enveloped viruses.\(^7,10\)

Low pH exposure is the second virus inactivation step where both enveloped and non-enveloped viruses are inactivated in 37°C at pH4 for 24 h.\(^1,7\)

The following technologies can be used for purification of IVIg products:

Ethanol fractionation, caprylate precipitation, Polyethylene-Glycol (PEG) precipitation, anionic exchange chromatography (AEC) (non-IgG plasma proteins and high molecular aggregates of IgG are removed), cationic exchange chromatography (CEC), size exclusion chromatography (SEC) (separation of IgG monomers, dimers and polymers), hydrophobic charge induction chromatography (HCIC), affinity chromatography and preparative electrophoresis.\(^13\)
2.5 The Complement System

The role of the complement system to defeat pathogens has been known for a century.\(^{14}\)

The complement system consists of about 20 proteins, which are present in the blood and in the extracellular fluid. The liver produces most of them.\(^{15}\)

The complement proteins interact with each other and are activated sequentially, often by antigen-antibody complexes on bacterial cells or by other mechanisms where specific antibodies are not involved. The complement proteins may cause lysis of bacterial cells, or, in other cases, labelling of the antigen-expressing cells. The latter stimulates macrophages, which leads towards a more effective destruction of the antigen.\(^2\)

The role of complement proteins is to eliminate infectious microorganisms and antigens from tissue and blood. This is possible due to a targeting mechanism accomplished by the complement system, allowing C3 receptor cells, for example phagocytic cells, to recognize and destroy the labelled agents.\(^{16}\)

When fragments of complement protein C3 bind to a pathogen, C3 is converted to two units, C3b and C3a. The activated form of C3, C3b binds covalently to the surface of the pathogen.\(^{16,17}\)

C3b is able to recruit fragments of other complement proteins when attached to the pathogen's surface. That enables a complex of complement proteins to be formed. The complex acts as a catalyst by initiating the subsequent steps in the proteolytic complement cascade. C3b is also acting as a marker when attached to the pathogen's surface, as phagocytic cells recognize the molecule, and are recruited to the site of infection for destruction of the pathogen.\(^{15}\)

The critical step for all activation pathways is the activation of complement protein C3. An individual with malfunctioned C3 protein is repeatedly having bacterial infections.\(^{15}\)

C3 and other activating components, initiating the complement cascade, are called proenzymes. When a proenzyme is cleaved, serine protease is generated, which is able to cleave the next proenzyme in the series. This activation-cleavage procedure is an amplifying proteolytic cascade. Each activated enzyme cleaves many molecules of the next proenzyme in the chain.\(^{15}\)

2.5.1 The Classical Pathway

The classical pathway is initiated through binding of IgG or IgM to antigens on cell surfaces. The bound antibodies fix complement proteins and the sequential cascade leading to destruction of pathogens is about to begin. The activating cascade is presented below.\(^2\)

1. Initiation: An antibody is binding to an antigen, forming a complex.
2. C1 components (C1q, C1r and C1s) bind to the antigen-antibody complex. C2-C4 bind to an adjacent membrane site and C3 is activated.
3. C3 membrane binding is catalyzing formation of a C5-C6-C7 complex at another membrane site.
4. Finally, C8 and C9 are fixed to the membrane leading to a pore formation and cell lysis.\(^2\)
The complement proteins C5 through C9 are together called the membrane attack complex (MAC) due to its role to form a pore in the cell membrane, causing lysis of the cell.\textsuperscript{2}

As a result of the complement activation cascade, by-products, so called anaphylatoxines are produced. The by-products serve as chemoattractants, activating phagocytes, which results in increased phagocytosis.\textsuperscript{2}

### 2.5.2 The Alternative Pathway

The alternative pathway is induced non-specifically. Serum proteins that are not involved in the classical pathway activate this pathway. The alternative pathway is presented below.\textsuperscript{2}

1. Properdin (P), a serum protein, is binding to the cell surface.
2. C3B (a complex of C3 and serum protein factor B) is fixed to P. The C3BP complex activates C5 catalyzes the formation of the MAC, which in turn results in cell destruction.\textsuperscript{2}

As the alternative pathway is less selective in comparison with the classical pathway, it can be activated by for example aggregated macromolecules such as aggregates of immunoglobulins and immune complexes. In this case, the reaction leads to a solubilisation of the complex due to a decreasing force between the antibodies as a result of covalent binding between C3b and the complex.\textsuperscript{15}

What complement activation pathways all have in common is that C3b triggers formation of the MAC complex which forms the aqueous pore in the membrane, making it leaky and in some cases causes the cells to lyse.\textsuperscript{15}

C3b is able to bind to both host cells and pathogens. Host cells are prevented from complement cascades on their surfaces by production of some special proteins and are therefore protected from destruction.\textsuperscript{15}

### 2.6 Anticomplementary Activity (ACA)

When large pools of plasma are mixed, up to 40\% of the Ig molecules spontaneously dimerize.\textsuperscript{19} Also, storage time and product formulation may increase aggregate formation.\textsuperscript{20}

Critical steps during the manufacturing process of Octagam\textsuperscript{®} are believed to influence on the characteristics of the IgG molecule:\textsuperscript{21}

- **pH adjustments**: The IgG molecule may be denatured if the NaOH or HCl concentration is too high, if the stirring speed is too low or if the diameter of the stirrer is too small.
- **Ultra/Diafiltration**: Membrane area, protein concentration, flow rates, transmembrane pressure and membrane regeneration procedure have influence on the molecule.
- **Chromatography**: Triton-X 100 binds to the resin and the IgG molecules are passing through. Flow rate, pressure, regeneration procedure etc. have influence on the molecules.
- **Final formulation**: Adjustments of pH, osmolality and protein concentration are parameters affecting the IgG molecules in the final container.\textsuperscript{21}
It is important to take all the critical parameters into consideration as the IgG molecule is sensitive and may be digested by enzymes, separate into heavy and light chains or form aggregates with other IgG in the manufacturing procedure of Octagam®. Aggregated IgG with complement binding activity is proposed to be one possible reason to adverse reactions that may happen to patients receiving intravenous immunoglobulins. The IVIg aggregates possess the ability to activate the complement system due to their complement binding activity, the anticomplementary activity, ACA. Therefore, the ACA of IVIG preparations is important to determine. Both the physical characteristics and the concentration of aggregates in IVIg preparations have influence on ACA.

Upon complement activation by the IgG aggregates, complement proteins C3 and C4 bind covalently to the IgG complexes. Covalent bonds between IgG and C3 are necessary for inhibition of immune precipitation and for the solubilisation of immune complexes. The covalent bond formation initiates the alternative pathway.

Not only aggregates of IgG have the ability to activate complement but also several isotypes of immunoglobulins do. IgM and the IgG subclasses IgG1 and IgG3 activate complement efficiently, meanwhile for example IgG2 is only effective when present in high doses and IgG4 is ineffective.
3 Assay Build-Up

The determination of ACA comprises three parts:

- Titration of haemolysin
- Titration of complement
- Determination of anticomplementary activity (ACA) in controls and samples of Octagam®

To perform determination of ACA in Octagam®, one must first have performed the two titrations above, in order to have proper dilutions of complement and haemolysin for the assay. The assay is usually not performed the same day as the titrations.

The titrations for the assay resemble the assay for determination of ACA in controls and samples, but the approaches differ a little.

3.1 Titration of Haemolysin

The haemolysin titration is performed to determine the optimal dilution of haemolysin (2MHU*/mL) for the titration of complement and for the determination of ACA in controls and samples. The optimal haemolysin dilution is determined from a diagram constructed from an approved dilution series of the haemolysin titration.

3.2 Titration of Complement

Titration of complement is normally performed once a month in routine, when a new batch of sheep blood is introduced. The outcome of the complement titration is the titer of the used complement with the haemolysin dilution of choice and the batch of blood used.

Out from the complement titer, the complement is in the ACA assay diluted to the activity of 100 CH50†/mL.

3.3 Determination of ACA in Controls and Octagam® 5% Samples

The ACA result of controls and samples depends on the haemolysin titration as well as the titration of the complement.

When ACA of immunoglobulins is determined, a defined amount of test material and a defined amount of guinea-pig complement are incubated together. The remaining amount of complement is titrated and is determined after incubation with haemolysin sensitized sheep

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* 1MHU = One minimal haemolytic unit in 1.0mL. This is the dilution of haemolysin such that further increase in the amount of haemolysin does not cause appreciable change in the degree of haemolysis.
† 1 CH50 = The haemolytic unit of complement activity. The amount of complement that, in the given reaction conditions will produce lysis of $2.5 \times 10^8$ out of a total of $5 \times 10^8$ optimally sensitised red blood cells.
red blood cells. Eventually, the haemolysis of the dilution series is measured spectrophotometrically at 541nm.

Four different controls are required to be included for each determination of ACA in controls and samples of Octagam®:

1. Two complement controls (requirement for approval: activity 80-120 CH₅₀/mL)
2. One BRP negative control (requirement for approval: ACA 10-40%)
3. One BRP pos. control (requirement for approval: ACA 60-100%)
4. One In house control sample of Octagam® 5% (requirement for approval: ACA 0.44-0.79 CH₅₀/mg IgG, which corresponds to ~22-39%)

Furthermore, each sample’s test is valid only if the plot for the sample dilution series is a straight line between 15-85% haemolysis.

The reference method for determining ACA at Octapharma is based on chapter 2.6.17 in the European Pharmacopoeia 6.0.

### 3.4 Reagents, Materials and Equipment

In this thesis, the following reagents, materials and equipment have been utilized for titrations and determination of ACA in controls and Octagam® 5% samples.

**Reagents used:**

- Magnesium and calcium stock solution. 1.103g CaCl₂ (2·H₂O) (Merck, USA) and 5.083g MgCl₂ (6·H₂O) (Merck, USA) are dissolved and diluted to 25mL with Milli-Q® H₂O.

- Gelatine barbital buffer stock solution (GBBS). The reagent is prepared from gelatine solution (1.25g of gelatine (Becton, Dickinson and Company, USA) dissolved in 1000mL of Milli-Q® H₂O) and Barbital buffer stock solution (BBS) (41.5g NaCl (Merck, USA) and 5.1g Barbital sodium (Apoteket Produktion & Laboratorier, Sweden) are dissolved in 800mL of Milli-Q®, pH is adjusted to 7.3 with 1M HCl and 2.5mL of magnesium and calcium stock solution is added).

GBBS is a mixture of one volume of BBS and four volumes of gelatine solution.

- Stabilised sheep blood (SRBC). Sheep blood is preserved in citrate solution as anticoagulant. (Statens veterinärmedicinska anstalt (SVA) or occasionally Siemens AB, Sweden (the latter was only used for the test of different sheep blood suppliers, chapter 4.3.11 and 5.2.11)

- Haemolysin (Ambozeptor). Antiserum against sheep red blood cells (SRBC), prepared in rabbits. (Siemens AB, Sweden)

- Guinea-pig complement. A pool of serum from the blood of at least 10 guinea pigs. (Charles River Laboratories, Germany)
Materials and equipment used:

- Milli-Q® H₂O (Millipore, France)
- Glass tubes: Test tube soda glass. 75 x 12.00 x 0.8-1.0mm (VWR, Sweden)
- Polystyren tubes: Ellerman 12 x 70mm (Nolato, Sweden)
- 96 well microtiter plate: Immulon® 1B plate (Thermo Scientific, USA)
- 96 well microtiter plate: Immuno 96 MicroWell™ plate (Nunc™, USA)
- Microtiter plate spectrophotometer: SpectraMAX 340 (Molecular Devices, USA)
- Spectrophotometer: Beckman DU-640 UV/VIS (Beckman Coulter AB, Sweden)
- Laboratory robot machine: Genesis RSP 150 (Tecan, Switzerland)
- pH-meter Mettler Delta 345 (Mettler Toledo AB, Sweden)
- Water bath: Model No. 1003 (GFL, Denmark)
- Air incubator: Heraeus® (Gallenkamp, UK)
- Centrifuge: Multifuge® 3S/3S-R (Kendro, Germany)
- Crushed ice 0°C and refrigerator 2-8°C
4 Methods and Performance

In this thesis parameters that are possibly critical to the determination of ACA in Octagam® are screened and two performances available for determination of ACA are compared.

Ideas for the experiments performed have come up partly on the basis of the change suggestions in table 1 and discussion between the Octapharma laboratories, partly by own initiative according as more has been understood about the ACA method and its peculiarities.

The two performances compared are the plate and the modified test tube method (refer to table 1, chapter 1). Some experiments have, where parameters have been studied, been performed with the modified test tube method, some with the plate method due to sharing of laboratory resources. The manual test tube performance has not been a part of the study.

One structural factorial design for the whole study would have been difficult to set up for the thesis. This is due to that there are many uncontrollable parameters influencing on the end result of the ACA assay as well as titrations. Consequently a couple of parameters have been studied one by one, or in one case, in a multiple regression analysis.

Some tests have often been performed only a few times, which means that the results of some studies should not be settled before more investigations under the same conditions have been done. The "hands-on approach" has resulted from the strapping for publications about the topic. Raw data from earlier performances as well as thesis results have been used to draw conclusions.

Variations within the assay like time and day-to-day variations have been unavoidable. As a biological system is used in the ACA assay as well as titrations, it is impossible to control many of the parameters involved. Reagents used are assumed to change a little in characteristics from day to day. This has not been taken into consideration for any of the experiments but when the variability has been tested. Complement and BRP control material were stored in -60°C and were therefore presumed not to vary in quality during the time for the thesis. Factors probably influencing on the ACA assay are listed in table 2.

All assays have been performed according to the Master Standard Operating Procedure (Master SOP) at Octapharma, which is based on the reference method for test of anticomplementary activity in immunoglobulins, presented in the European Pharmacopoeia24. A validated template for calculation has been used for calculation of ACA results as well as for complement and haemolysin titration results.

The following formulas have been used directly or indirectly in the template (retrieved from the European Pharmacopoeia24):

The degree of haemolysis (Y) is calculated according to the formulae:

\[ \frac{A_a - A_f}{A_b - A_f} \times 100 \quad [1] \]

where:

- \( A_a \) = Absorbance of a supernatant in the dilution series
- \( A_b \) = Mean absorbance of supernatants constituting 100% haemolysis
\[ A_t = \text{Mean absorbance of supernatants constituting 0\% haemolysis} \]

The activity in haemolytic units (CH\(_{50}\)/mL) is calculated according to the formulae:

\[
\frac{C_d}{C_a \times 5} \quad [2]
\]

\( C_d = \text{Reciprocal value of the complement dilution} \)

\( C_a = \text{Volume of diluted complement in millilitres resulting in 50 per cent haemolysis} \)

5 = Scaling factor to take account of the number of red blood cells

ACA of the BRP control (\%) is calculated according to the formulae:

\[
\frac{a - b}{a} \times 100 \quad [3]
\]

\( a = \text{Mean complement activity (CH}_{50}\)/mL of the complement control} \)

\( b = \text{Complement activity (CH}_{50}\)/mL of the tested sample} \)

To obtain the ACA in CH\(_{50}\)/mg IgG (the consumption of complement by one mg of IgG), formulae [3] is used and the percentage value is divided by the IgG concentration in the sample.

Statistical tests in this thesis have been performed using SPSS 17.0 and Microsoft Office Excel XP.

### 4.1 Parameter Overview and Delimitations

It has earlier been demonstrated that the Relative Standard Deviation (RSD) of the plate method for determination of ACA is 25\%. This value has been calculated from intermediate precision studies, when parameters involved in the ACA assay have been unintentionally shifted from occasion to occasion. The high method dispersion illuminates the meaning of repeating each test many times to ensure the correctness of the results in the current circumstances. In this case, though, the time for the thesis was delimited and just a few tests for each change were done. This rendered an overview of which factors could possibly be critical to the assay.

Not only are the parameters of the ACA assay itself affecting the outcome. Also titration performances and interpretation of titration results are assumed to have influence on ACA. A lot of controllable as well as uncontrollable parameters are thought to have impact on the end result. Also interaction effects between parameters could be critical to the ACA, since the system used for the determination is a complex biological system. The system varies in
properties from batch to batch of haemolysin, complement and SRBC – components that behave differently at different analytical circumstances. The ACA assay takes about five hours to perform and therefore time variations occur. The many steps involved in the assay make it impossible to perform all assays completely identically.

Table 2 represents some parameters that probably are critical to the ACA assay as well as the titrations. Not all of them have been tested in this thesis.

Table 2. A Set of Parameters Defined as Critical for the ACA Assay and Titrations. The table presents parameters defined as critical to the outcome of the ACA assay and titrations. The parameters were stated during the thesis. Comments describe each parameter in short and why it is assumed to be critical.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Which method that is applied</td>
<td>Surface to volume area differs between the plate and the tube method. Volumes in a microtiter plate are smaller than the volumes used for incubation in test tubes. When applying plate performance, a 37ºC air-filled incubator is used for the incubation, whereas for test tubes, a 37ºC water bath is used for the purpose. For plate performance, the plates are cooled in refrigerator after the second incubation. When applying the test tube method, an ice bath is used for the cooling down procedure. Direct contact with cool or warm water results in a more efficient heat transmission than air contact.</td>
</tr>
<tr>
<td>Analyst</td>
<td>The result may vary for different laboratory technicians due to different pipetting techniques, handling of samples, time durations etc.</td>
</tr>
<tr>
<td>Time variations within the assay</td>
<td>The assay takes about 5 h to perform and the time for different steps within the assay varies from time to time.</td>
</tr>
<tr>
<td>Day to day variations</td>
<td>Shifting of characteristics of reagents between different days may have influence on the ACA.</td>
</tr>
<tr>
<td>Time of cooling procedures</td>
<td>At least 5 min are required for cooling down the incubated material after the two incubations, but no upper limit of the cooling procedures is specified. This means that the ACA may vary depending on how low the temperature of the incubated mixtures gets.</td>
</tr>
<tr>
<td><strong>Temperature of the GBBS</strong></td>
<td>The GBBS in the assay may be used RT or cooled down. When cooling down the buffer before it comes in touch with complement, the temperature of it varies more from assay to assay than the RT buffer. This is due to time variations in the assay, which affect how long time the buffer can be cooled. The GBBS buffer have to be prepared the same day as the analysis is performed so it is not possible to let it stand in refrigerator over night.</td>
</tr>
<tr>
<td><strong>pH of the GBBS</strong></td>
<td>The pH of the GBBS has to be within the range 7.25-7.34. If the GBBS once is 7.25 and 7.34 the next time, the sensitive biological system may be affected during analysis.</td>
</tr>
<tr>
<td><strong>pH of controls and samples</strong></td>
<td>The pH of controls and samples may vary between 6.8 and 7.0 when adjusted.</td>
</tr>
<tr>
<td><strong>Batch of haemolysin</strong></td>
<td>Characteristics may vary between batches.</td>
</tr>
<tr>
<td><strong>Batch of complement</strong></td>
<td>Characteristics may vary between batches.</td>
</tr>
</tbody>
</table>
| **Blood quality** | The quality of the blood may vary between each blood tap, especially if two different batches of blood are from two different sheep donors. If blood is tapped from several sheeps and is pooled, the robustness of the blood should be higher resulting in increased precision of the method.  
The blood is also assumed to change in characteristics during the time of storage (in 2-8°C for up to three weeks) since it is biological material. |
| **Dilution of haemolysin in the ACA assay** | The optimal dilution of haemolysin (2MHU/mL) is determined from the haemolysin titration. The result may be difficult to interpret and subjective interpretations result in different haemolysin dilutions in the ACA assay depending on who does the interpretation. |
| **Complement activity in the ACA assay** | Since the complement activity is allowed to vary between 80-120 CH₅₀/mL, the ACA of controls and samples may vary as well. |
| **Predilution of the complement for the BRP pos. control** | The predilution of the complement for the BRP pos. control could be either 1:50 or 1:100, which may give different outcomes of the BRP pos. control. |
| **Cleanness of blood after washing procedure** | How well the blood is washed varies from time to time due to variations of the blood quality of the day, how balanced the centrifuge is during the washing procedure, for how many times the wash is performed etc. |
The freshness of the 5% SRBC and SRBC-S suspensions

Time variations within the assay make it difficult to have complete control over how long the 5% SRBC and the haemolysin-coated SRBC (SRBC-S) stay before usage.

Incubated volumes of diluted haemolysin and 5% SRBC for preparation of SBRC-S

The volumes should be equal, but the amount of each preparation to be incubated is not stated in the European Pharmacopoeia. A large volume takes longer time to warm up than a low one. Incubation is performed in a 37°C water bath.

4.2 Raw Data Studies

Raw data was collected to look at relations between parameters that could explain different outcomes of the BRP pos. control. At first, the BRP pos. control was plotted over a period to look at trends. The raw data for the plot was collected from runs where the test tube method had been applied.

The BRP pos. control was also plotted against the In house control, to look at the relation between them. Data was taken from 18 consecutive assays performed with the tube method and 18 consecutive assays performed with the plate method. The relations were plotted for respective method.

Furthermore, 31 BRP pos. control results from ACA assays performed with the tube method were compared to 31 BRP pos. control results from ACA assays performed with the plate method. Statistical analysis of the results was performed in Microsoft Office Excel XP in order to compare the two methods.

4.3 Robustness Testing

The robustness is a measure of the method's capacity to remain unaffected by small variations in method parameters.25

4.3.1 Temperature Measurements of Cooling Procedures in Fridge versus on Ice

When the plate method is applied for ACA determination, the second incubation is followed by a cooling procedure in a 2-8°C fridge for at least 5 min in order to stop haemolytic reactions. To stop the reactions, 5 min of cooling may be insufficient.

Temperature measurements were performed to get an overview of the temperature decrease by time. All wells in two Immuno 96 MicroWell™ plates (Nunc™, USA) were filled with 300 µL of Milli-Q®, the same volume as used for the incubation in the titrations and the ACA assay, when the plate method is employed. The volumes were incubated for 1h in a 37°C air-filled incubator before the temperature measurements were performed. Temperature decrease by time was measured on ice for one of the plates and in a 2-8°C fridge for the other.
4.3.2 Drifting of Absorbance of 5% SRBC + Milli-Q® H₂O

5% SRBC (1mL washed SRBC diluted in approximately 20mL of GBBS) can be prepared in advance before it is used for incubation with diluted haemolysin for preparation of SRBC-S. Preparation in advance facilitates the method performance for the analyst. To look at differences between storage in 2-8°C and in RT for old and new blood, absorbances were measured with even time intervals after preparation of the 5% SRBC suspension. For each absorbance measurement, 0.2mL of 5% SRBC and 2.8mL of Milli-Q® were vortexed together, getting full haemolysis of the red blood cells in the mixture. One tube of 5% SRBC were stored in 2-8°C and one in RT. Absorbances were measured once at 541nm using Beckman DU-640 UV/VIS Spectrophotometer (Beckman Coulter AB, Sweden). The GBBS volume for the 5% SRBC suspensions was initially adjusted so that the first reading resulted in roughly 0.62 AU.

The cell density in the 5% SRBC suspension is suitable if the mixture of 5% SRBC and Milli-Q® at 541nm is 0.62 ± 0.01.²⁴

4.3.3 Comparison of Complement Titers - Difference between Plate and Test Tube Performance

Differences in the complement titer comparing complement titration results in test tubes and on microtiter plates have earlier been indicated. To confirm the result, complement titrations were performed in test tubes and on microtiter plate respectively.

Plate and test tubes as well as chilled and RT GBBS were respectively compared where the same batch of blood had been used. The chilled GBBS used with plate performance in some of the assays was a deviation from the natural plate performance.

Master thesis results were together with raw data used as material for paired two-sample test of means on a significance level of 5%, in Microsoft Office Excel XP. The null hypothesis and the alternate hypothesis for the one-tail test were stated as follows (µ₁ = mean complement titer for plate performance and µ₂ = mean complement titer for test tube performance):

H₀: µ₁ = µ₂  
(There is no difference in complement titer comparing performance on plate and in test tubes.)

H₁: µ₁ > µ₂  
(Complement titers when titration is on plate are determined higher than when the test tube performance is applied.)

The blood batch used for each titration was not regarded when the t-test was performed. However, the sample size was 10 for each performance. Therefore differences due to blood quality were assumed to not affect the outcome of the test considerably.

Statistics used for the t-test is presented in appendix A.
4.3.4 Parameter Influence on the Complement Titer, Plate Performance

The Immulon® 1B plate (Thermo Scientific, USA) used for incubation in the context of the plate method is of medium binding character, a property that might have influence on the reactions in the incubation mixtures. Since incubations for the test tube methods are in non-treated plastic tubes, it was of interest to evaluate if a non-treated Immuno 96 MicroWell™ plate (Nunc™, USA) gave different results compared to the corresponding plate with medium binding properties, Immulon® 1B.

A total of four complement titrations on each plate were conducted under the same conditions.

One of each plate was incubated at every complement titration. Changes due to days since opening of the blood container were included in the model. Another factor taken into account was which plate was first used in the Genesis RSP 150 laboratory robot since the time for transfer might have had influence on the end result. The third factor was which plate that gave the result. The multiple regression model describing the complement titer outcome is presented in Table 3. The model is assumed to be linear.

Plate disparities were not to be studied for the ACA assay due to time limitations. The ACA assay itself is, except from the complement titration, also including antibodies, which may behave differently in a plate with binding properties compared to a non-treated plate. The binding plastic in the wells may have complement fixating capabilities increasing the ACA.

Table 3. Multiple Linear Regression Model for Prediction of the Complement Titer.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description of the variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y$</td>
<td>Complement titer (CH50/mL)</td>
</tr>
<tr>
<td>$z_1$</td>
<td>=1 when Immulon® 1B medium binding plate is used, else 0</td>
</tr>
<tr>
<td>$z_2$</td>
<td>=1 when the Immulon® 1B plate is used first in Genesis RSP 150, else 0</td>
</tr>
<tr>
<td>$\beta_0$</td>
<td>Describes the mean complement titer</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>Describes the difference between the two plates</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Describes the difference between time point 1 and 2</td>
</tr>
<tr>
<td>$\beta_3t$</td>
<td>Describes the drift by time</td>
</tr>
<tr>
<td>$t$</td>
<td>The time (in days) since the container of blood was opened</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Random error term</td>
</tr>
</tbody>
</table>
4.3.5 Soundness of the Haemolysin Titration, Test Tube Performance

To date, haemolysin titration is performed for every new batch of haemolysin. Differences in the result of haemolysin titrations comparing two different batches of complement were studied. Haemolysin titrations with the two different batches of complement were performed once for each on two consecutive dates. The titration results were compared.

4.3.6 Potential Differences due to Different Batches of Haemolysin and Complement, Test Tube Performance

Other complement and haemolysin batches than at the moment used for ACA were tested with the modified tube method. This was done in order to see how the BRP pos. control was affected by another combination of batches. When the tested batches have been used for ACA with the manual test tube method, the BRP pos. control has been approved.

The test was performed with the same haemolysin dilution as was used for the manual tube method at the moment. The complement for the BRP pos. control was prediluted 1:100 in the first run and 1:50 in the second.

SRBC from SVA were used for the test, which was not the case for the manual tube method, where Siemens AB is the supplier of choice.

4.3.7 Impact on ACA by Different Haemolysin Dilutions

Different haemolysin dilutions were tested in the ACA assay and the impact on the results of the BRP pos. control and the In house control was studied. Batches of blood, complement and haemolysin were held constant for all assays (batch of blood in all assays but two) to exclusively look at the variations due to the different dilutions of haemolysin.

4.3.8 Impact on ACA by Different Complement Activities

How to dilute the complement for the ACA assay is determined by the complement titration. The span for requirement of the complement activity in the ACA assay is quite wide (80-120 CH50/mL) which allows the complement to be diluted with different diluting factors, still getting approved complement controls for the ACA. The effect of different complement activities was studied. The relations between the complement activity and the BRP pos. control as well as with the In house control were looked at.

4.3.9 The Effect of Different Predilutions of the Complement for the BRP Pos. Control, Test Tube Performance

Different predilutions of the complement for the BRP pos. control were studied.

The complement for the BRP pos. control is prediluted differently in comparison with the other controls and samples.

Predilution 1:50 and 1:100 were tested to see how the BRP pos. control was affected. The study was performed with the test tube method.
4.3.10 Comparison of ACA Methods: Plate versus Tube Performance

Two ACA assays, one on plate and one in test tubes, were performed the same day. This was in order to have a glance at if the plate and tube methods for determination of ACA in controls and samples resulted in different ACA. By doing the tests the same day, day-to-day variations could be eliminated. As one analyst only is able to perform one ACA a day, two different analysts ran the two methods. This means that the human factor could not be eliminated.

4.3.11 Different Sheep Blood Suppliers, Test Tube Performance

Siemens AB pool their blood from several sheeps instead of tapping the blood from only one sheep as SVA. By pooling blood from several sheeps, it is intuitive that the precision of the complement titration as well as of the ACA should increase, giving more similar results from time to time.

When the batches of haemolysin and complement were tested (refer to chapter 4.3.6 and result in 5.2.6), SRBC from SVA were used in contrast to the manual tube method, where Siemens AB supplies SRBC. As the BRP pos. control was not approved with the modified test tube method when SVA blood was used, it was of interest to test SRBC from Siemens AB.

Sheep blood from Siemens and SVA were tested in order to find out how different blood properties would influence on the BRP pos. control results for the tube method.

Three ACA runs of the pooled blood were performed, two with blood batch 3030248 and one with blood batch 3030249 (Siemens AB). The non-pooled blood from SVA used in routine was once tested in parallel.
5 Results and Discussion

5.1 Raw Data Studies

5.1.1 BRP Pos. Control Results, Test Tube Performance

The trend line for BRP pos. control results (18 consecutive results), determined by performance of ACA in test tubes, is presented in fig. 1. As can be seen, the frequency of non-approved controls, e.g. controls <60% in ACA, is high.

Percentage of non-approved BRP pos. controls during the period was (8/18)*100 = 44%

Deviating patterns in fig. 1 can be seen:

- Difference between assay occasion 9 and 10: At analysis 9, sheep ID 4068 was used for blood and at analysis 10, sheep ID 302 was used. 4068 has been used as blood donor for most of the assays in fig. 1.
- Assay occasion 11: Blood from sheep 4068 was again used. Therefore, the tip at occasion 10 may have been caused by another quality of the blood in sheep ID 302, resulting in a higher BRP pos. control.
- Dip at assay occasion 15: Sheep ID 7005 was used as donor for the testing. The sheep ID had never been tested before.
- No explanation could be found to the BRP control result at assay occasion 17 (sheep ID 4068). In comparison to, for instance assay occasion 11, where also sheep ID 4068 was used, it is a rather high result. The difference may be due to that the sheep’s condition changed between the two occasions the two batches were drawn.
- Dip at assay occasion 18: A new tester for ACA performed the analysis. Hence, it can be presumed that some extent of training is needed to get the BRP pos. control within limits. Duration of the steps in the assay might have had caused unwanted biological reactions, which in turn leads to a low control.

As can be figured out from the deviating patterns in the trend line, the batch of sheep blood, especially the sheep donor seems to affect which BRP control result that is achieved. The skills of the analyst also seem to influence.
Fig. 1. Trend Line for the BRP Pos. Control, Determined in Test Tubes. ACA results of the BRP pos. control were plotted over a randomly chosen period to look at the frequency of non-approved controls. 18 consecutive results are presented in the graph. The number of non-approved controls during the period was estimated to 44%.

5.1.2 Relation between the In House Control and the BRP Pos. Control

A higher BRP pos. control result should, in theory, imply a higher In house control result and vice versa. The relation between ACA of the In house control of Octagam® 5% and the BRP pos. control for performance in test tubes (see fig. 2), displays a decreasing trend. However, this is not clear if the trend line is not added as the dots are scattered in the plot. It does not seem to appear any positive correlation pattern even though the outlier in the graph is excluded.

When looking at the comparison with plate performance in fig. 3, the dots are scattered and the two controls show no linear ascending correlation. However, if the three outlying dots are excluded from fig. 3, a positive correlation appears. Presuming that this is the true relationship, the plate method is preferable due to its better reliability of the BRP positive control.

The odd appearance in fig. 2 is believed to depend on the BRP control and not the In house control. This statement is based on earlier experience of the BRP positive control. The In house control can be considered to be more representative to the samples than the BRP reference since the In house control is an Octagam® sample and is similar to the samples analysed.
Fig. 2. Relation between the In House Control and the BRP Pos. Control, Test Tube Performance. Test tube performance has been applied for analysis. The 18 results are taken from 18 consecutive assays.

Fig. 3. Relation between the In House Control and the BRP Pos. Control Plate Performance. The values presented in the graph are 18 consecutive results from ACA assays performed with the plate method.

5.1.3 Comparison of BRP Pos. Control results Applying Either Plate or Test Tube Performance

The comparison between 31 BRP pos. control results from ACA assays performed with the tube method and 31 BRP pos. control results from ACA assays performed with the plate method resulted in the graph in fig. 4. Statistical analysis results that were calculated using Microsoft Office Excel XP are presented in table 4.
Table 4 displays the calculated means, Standard Deviations (SD) and Relative Standard Deviations (RSD) of the data presented in fig. 4. Calculation formulas for SD and RSD are presented in appendix A.

As can be seen in table 4, the average ACA was >60% when applying the plate method and <60% for the test tube method. Hence, the average result for test tubes was not approved. The RSD was higher applying the test tube performance than the plate performance (7.11% compared to 11.7%), which can also be seen in fig. 4, the dots are more scattered for test tubes. The lower dispersion for plate indicates a better intermediate precision of that method.
5.2 Results, Robustness Testing

5.2.1 Temperature Measurements of Cooling Procedures in Fridge versus on Ice

The results for the temperature decrease in plate following the second incubation, comparing cooling in fridge and cooling on ice, are presented in fig. 5 and 6. When the fridge was used for cooling, the temperature reached 27°C after 5 min and 18°C after 14 min. When ice was used for cooling, the temperature reached 23°C after 5 min and 15°C after 14 min. The slopes of the fitted lines are \(-1.27^\circ\text{C/min}\) and \(-1.34^\circ\text{C/min}\) respectively. The steeper slope for ice indicates that samples possibly should be cooled on ice if they are cooled down only for five minutes. However, the curve for the fridge cooling procedure is more even compared to that of the on ice cooling procedure.

![Temperature Decline, Fridge Cooling](image)

\[ y = -1.2657x + 34.407 \]

Fig.5. Temperature decline after the second incubation when the plate was cooled in fridge. 4-8°C. The temperature reached 27°C after 5 min and 18°C after 14 min.
Temperature Decline, Ice Cooling

\[ y = -1.3421x + 31.342 \]

<table>
<thead>
<tr>
<th>Time after the beginning of the cooling procedure (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 6.** Temperature decline after the second incubation when the plate was cooled on ice. The temperature reached 23°C after 5 min and 15°C after 14 min.

### 5.2.2 Drifting of Absorbance of 5% SRBC + Milli-Q® H₂O

The results for the absorbance measurements of the 5% SRBC mixed with Milli-Q® are presented below (fig.7-fig.10). The data is to be found in appendix B. The largest gap between the lowest and highest absorbance was gained for the SRBC mixture 0 days after opening, when the mixture had been kept 150 min in 2-8°C (fig. 9).

**Fig. 7.** The graph represents the absorbance drifting for the SRBC and Milli-Q® mixture, kept in RT. Measurements were performed 0 days after opening of the blood container.
**Fig. 8.** The graph represents the absorbance drifting for the SRBC mixture kept in RT. Measurements were performed 15 days after opening of the blood container.

**Fig. 9.** The graph represents the absorbance drifting for the SRBC mixture kept in 2-8°C. Measurements were performed 0 days after opening of the blood container. A bigger difference in absorbance by time was observed for fresh blood than for 15 days old blood.

**Fig. 10.** The graph represents the absorbance drifting for the SRBC mixture kept in 2-8°C. Measurements were performed 15 days after opening of the blood container. A smaller difference in absorbance by time was observed for 15 days old blood than for fresh blood.

For three out of four measurement series above (all figures but fig. 9, 2-8°C, 0 days after opening), the absorbance is first increasing and then decreasing. Even though the
measurement series in fig. 9 behave differently, all measurements show that the blood stability is poor when it comes to the absorbance drifting of the 5% SRBC.

To be sure that the absorbance is the starting value for the ACA test, the 5% SRBC should not be prepared in advance but as close to the usage as possible.

5.2.3 Comparison of Complement Titers – Difference between Plate and Test Tube Performance

Results from the complement titrations performed during the thesis in order to study differences in complement titer altering assay performance and temperature of the GBBS are presented in table 5.

Table 5. The table represents results from complement titrations performed during the thesis with different batches of sheep blood, either applying the tube or the plate method, either performed with chilled or RT GBBS.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Date</th>
<th>Blood batch No.</th>
<th>Performance</th>
<th>Hemolysin batch No./diluted</th>
<th>Complement batch No.</th>
<th>GBBS chilled/RT</th>
<th>Complement titer (CH50/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2009-03-16</td>
<td>4068/090309</td>
<td>Test tubes</td>
<td>302180/1:500</td>
<td>9WA006642</td>
<td>chilled</td>
<td>226</td>
</tr>
<tr>
<td>2</td>
<td>2009-03-18</td>
<td>4068/090309</td>
<td>Plate</td>
<td>302180/1:500</td>
<td>9WA006642</td>
<td>chilled</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>2009-03-18</td>
<td>4068/090309</td>
<td>Plate</td>
<td>302180/1:500</td>
<td>9WA006642</td>
<td>RT</td>
<td>251</td>
</tr>
<tr>
<td>4</td>
<td>2009-03-20</td>
<td>4068/090309</td>
<td>Test tubes</td>
<td>302180/1:500</td>
<td>9WA006642</td>
<td>RT</td>
<td>226</td>
</tr>
<tr>
<td>5</td>
<td>2009-04-06</td>
<td>4068/090330</td>
<td>Test tubes</td>
<td>302180/1:500</td>
<td>9WA006642</td>
<td>chilled</td>
<td>213</td>
</tr>
<tr>
<td>6</td>
<td>2009-05-19</td>
<td>4068/090511</td>
<td>Plate</td>
<td>302180/1:800</td>
<td>9WA006642</td>
<td>RT</td>
<td>304</td>
</tr>
<tr>
<td>7</td>
<td>2009-05-19</td>
<td>4068/090511</td>
<td>Test tubes</td>
<td>302180/1:800</td>
<td>9WA006642</td>
<td>chilled</td>
<td>253</td>
</tr>
</tbody>
</table>

When run 1 is compared to run 2 and run 3 with 4, a higher complement titer is observed for plate than for tubes. This is when performance with same tempered GBBS has been used on plate and in test tubes and the same batch of blood has been used. From the titrations performed the same day (run 6 and 7), a higher complement titer was determined with the plate method. However, the GBBS was differently tempered for these two assays. Run 1 and 4 were performed with the same batch of blood, both of them in test tubes, and the complement titer was the same for both of them. If that result is true, it can be stated that complement titration with test tube performance is not affected by the temperature of the buffer. Out from that, the method of choice is the most critical to the complement titer. When run 2 and 3 are compared, it can be seen that the complement titer gets higher with cold GBBS on plate than with RT GBBS on plate.

Regarding these data, the highest complement titer would be gained with cold GBBS applying the plate method.

Raw data and all thesis data but run 5 (to compare equal number of tests for both methods) are listed in table 6. The GBBS has most often been chilled when test tubes have been used and RT when plate has been used for complement titration. Paired two-sample test for means comparing complement titer on plate versus in test tubes was performed using Microsoft Office Excel XP, see table 7. The t-test on the α-level 5% showed that complement titration on plate results in a significantly higher complement titer compared to complement titration in test tubes. This is because the test statistical value is bigger than the one-tail critical value. The null hypothesis, $H_0: \mu_1 = \mu_2$ could be rejected in favour of $H_1: \mu_1 > \mu_2$. 


The difference in complement titer means that complement has to be diluted differently for the two methods when ACA of Octagam® batches is analysed. Since it is the complement activity and not the titer that is involved in the ACA of the product (the complement is diluted to get an activity between 80 and 120 CH<sub>50</sub>/mL), the high complement titer determined on plate does not itself influence on the end result.

It can in table 6 be seen that the complement titer varies for different batches of blood, especially for different sheeps. For example sheep ID 7005 gave a remarkably lower complement titer at assay occasion 13, compared to the blood batches from sheep ID 4068 on assay occasions 12 and 14.

**Table 6.** Figures from raw data and from thesis complement titrations. Thesis results are presented in the last six rows of the table. The complement titer varies for each assay occasion and blood from sheep ID 7005 gave a remarkably lower complement titer (122 CH<sub>50</sub>/mL) compared to the blood of the others.

<table>
<thead>
<tr>
<th>Assay occasion</th>
<th>Method performance</th>
<th>Complement titer (CH&lt;sub&gt;50&lt;/sub&gt;/mL)</th>
<th>Sheep ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>plate</td>
<td>253</td>
<td>304</td>
</tr>
<tr>
<td>2</td>
<td>plate</td>
<td>258</td>
<td>4068</td>
</tr>
<tr>
<td>3</td>
<td>plate</td>
<td>264</td>
<td>4068</td>
</tr>
<tr>
<td>4</td>
<td>plate</td>
<td>226</td>
<td>301</td>
</tr>
<tr>
<td>5</td>
<td>plate</td>
<td>263</td>
<td>4068</td>
</tr>
<tr>
<td>6</td>
<td>plate</td>
<td>304</td>
<td>203</td>
</tr>
<tr>
<td>7</td>
<td>plate</td>
<td>257</td>
<td>4068</td>
</tr>
<tr>
<td>8</td>
<td>test tubes</td>
<td>202</td>
<td>4068</td>
</tr>
<tr>
<td>9</td>
<td>test tubes</td>
<td>206</td>
<td>4068</td>
</tr>
<tr>
<td>10</td>
<td>test tubes</td>
<td>203</td>
<td>4068</td>
</tr>
<tr>
<td>11</td>
<td>test tubes</td>
<td>254</td>
<td>302</td>
</tr>
<tr>
<td>12</td>
<td>test tubes</td>
<td>209</td>
<td>4068</td>
</tr>
<tr>
<td>13</td>
<td>test tubes</td>
<td>122</td>
<td>7005</td>
</tr>
<tr>
<td>14</td>
<td>test tubes</td>
<td>227</td>
<td>4068</td>
</tr>
<tr>
<td>15</td>
<td>test tubes</td>
<td>226</td>
<td>4068</td>
</tr>
<tr>
<td>16</td>
<td>plate</td>
<td>300</td>
<td>4068</td>
</tr>
<tr>
<td>17</td>
<td>plate</td>
<td>251</td>
<td>4068</td>
</tr>
<tr>
<td>18</td>
<td>plate</td>
<td>304</td>
<td>4068</td>
</tr>
<tr>
<td>19</td>
<td>test tubes</td>
<td>253</td>
<td>4068</td>
</tr>
<tr>
<td>20</td>
<td>test tubes</td>
<td>226</td>
<td>4068</td>
</tr>
</tbody>
</table>
Table 7. Paired t-test for means on a significance level of 5% showed that a significantly higher complement titer is achieved applying the plate method than when the test tube method is applied, as the test statistic value is higher than the one-tail critical value. The t-test was performed using Microsoft Office Excel XP.

<table>
<thead>
<tr>
<th></th>
<th>Plate</th>
<th>Test tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>268</td>
<td>213</td>
</tr>
<tr>
<td>Variance</td>
<td>684</td>
<td>1376</td>
</tr>
<tr>
<td>Observations</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>df</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>t Stat</td>
<td>6.623152291</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) one-tail</td>
<td>4.83342E-05</td>
<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.833113856</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>9.66684E-05</td>
<td></td>
</tr>
<tr>
<td>t Critical two-tail</td>
<td>2.262158887</td>
<td></td>
</tr>
</tbody>
</table>

5.2.4 Parameter Influence on the Complement Titer, Plate Performance

As can be seen in fig. 11, the relation between the complement titer and which plate generating the titer is a weak negative relationship. The complement titer for the plate with binding properties is slightly lower than the complement titer for the non-binding plate. Only 1.1% ($R^2$ Linear = 0.011) of the variability of the complement titers earned could be explained by the variability of the plate generating the titer. Therefore, the binding properties of the plate are not assumed to be critical to the determination of the complement titer.

Fig.11. Relation between the Complement Titer and the Plate Generating the Titer.
The correlation is negative but weak. Therefore, the binding property of the plate is not critical to the determination of the complement titer.
When looking at the relation between the plate used first in Genesis RSP 150 and the titer found, a weak positive relationship was found, see fig. 12. The titer is slightly higher for the Immulon® 1B plate than for the Immuno 96 MicroWell™ plate. As only 1.5% of the variability of the points earned can be explained by the variability of the plate used first in Genesis RSP 150, the matter of which plate that is transferred to first in the robot seems to be of minor importance for the complement titer.

![Fig. 12. Relation between the Complement Titer and the Plate used First in Genesis RSP 150. The correlation is positive but quite weak. Therefore, the plate used first in the robot is not critical to the determination of the complement titer.](image)

The relation between the complement titer and the time since the blood container was opened, is clearly linear, see fig. 13. Here, a strong negative linear relationship was found. 69.5% of the variability of the complement titers earned could be explained by the variability of time in days since the blood container was opened. The longer the blood is stored, the lower the complement titer gets.
A linear multiple regression model was assumed but the only parameter that can be concluded to be linear is the parameter “time since the blood container was opened”, as it is continuous. The other parameters only have two levels and therefore, the appearance in between is not seen.

The SPSS output of the multiple linear regression is presented in appendix C.

The variables used in the model predict the complement titer well as the R value (the multiple correlation coefficient) is 0.847 in the SPSS output, which is close to 1. As the R square is 0.718, 71.8% of the variance in complement titer can be predicted by the combination of the three independent variables used in the model.

As can be seen in appendix C, the p value (Sig.) of the model is 0.134 which is lower than 0.15 but not than 0.10. This means that the model is useful on a significance level of 15%, but not on a level of 10%.

As can be predicted from the coefficients in the output, the regression equation describing the model is:

\[ \hat{y} = 290.409 - 1.734z_1 - 1.973z_2 - 4.001t + \varepsilon \]

T-tests were performed on a significance level of 5% to test the null hypothesis that each independent variable not had a significant relationship with the dependent variable (complement titer).
H₀ : \( \beta_i = 0 \) for all \( i = 1, 2, 3 \)

H₁ = \( \beta_i \neq 0 \) for at least one \( i \)

If the null hypothesis is tested on a significance level of 5% for each \( i \), only the \( t \) variable “time since the blood container was opened” is significantly contributing to the dependent variable (the complement titer). Therefore H₀: \( \beta_3 = 0 \) can be rejected. The variables \( z_1, z_2 \) (the plate generating the titer and the plate used first in the robot) do not contribute significantly to the complement titer.

5.2.5 Soundness of the Haemolysin Titration, Test Tube Performance

The two haemolysin titrations tested resulted in the graphs in fig. 14 and 15. The highest haemolysis degree was between 50 and 70% for the two approved series, which is a requirement for approval. For haemolysin batch No. 302180 and complement batch No. 9WA006642, the complement dilution for approval was 1:225 (fig. 14) and for complement 8WA036798, the complement for the approved dilution series was diluted 1:200.

As can be seen in fig. 14 and 15, the profiles differ between the complements, which means that the optimal haemolysin dilution for the ACA assay, interpreted from two diagrams will differ. Therefore, a new haemolysin titration should be performed not only for every new batch of haemolysin, but also for every new batch of complement.

**Fig.14.** Approved plateau for haemolysin 302180 and complement 9WA006642. The complement dilution for approval was 1:225. The assay was performed 2009-02-27.
Further on, a new batch of complement means a new biological component to the biological system presumably not interacting identically as the former used batch of complement. This argues for a new haemolysin titration for every new batch of complement.

5.2.6 Potential Differences due to Different Batches of Haemolysin and Complement, Test Tube Performance

The ACA test in the first run, when the complement was prediluted 1:100 resulted in a non-approved BRP pos. control (50%).

The second run, when the complement for the BRP pos. control was prediluted 1:50, the BRP pos. control was still non-approved with an ACA of 51%.

One obvious difference between the complement dilutions was observed; The haemolysis degree increased much faster with dilution 1:50 compared to dilution 1:100 and resulted in just a few approved points between 15-85% haemolysis in the graphs for the dilution series. This is confirmed in section 5.2.9.

The SRBC supplier was after this test suspected as critical and was tested, see chapter 4.3.11 and 5.2.11.
5.2.7 Impact on ACA by Different Haemolysin Dilutions

The results from the haemolysin dilution comparisons are viewed in table 8. The average results from the two haemolysin dilutions 1:500 and 1:800 are presented in table 9. The two different batches of blood were not taken into account for the average calculations.

Out from the data in table 8, it can be seen in table 9 that the average of the BRP pos. control is higher for the 1:800 haemolysin dilution than for the 1:500 dilution for both methods. The In house control is also higher for the 1:800 dilution when determined with the plate method.

Table 8. Results of the BRP pos. control and the In house control, comparing two haemolysin dilutions.

<table>
<thead>
<tr>
<th>Assay occasion</th>
<th>Hemolysin dilution</th>
<th>Performance</th>
<th>Batch of blood</th>
<th>BRP pos. control (%)</th>
<th>In house control (CH50/mg IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:500</td>
<td>Plate/cold GBBS</td>
<td>4068/090309</td>
<td>60</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>1:500</td>
<td>Plate/cold GBBS</td>
<td>4068/090309</td>
<td>66</td>
<td>0.36</td>
</tr>
<tr>
<td>3</td>
<td>1:500</td>
<td>Plate/cold GBBS</td>
<td>4068/090309</td>
<td>46</td>
<td>0.49</td>
</tr>
<tr>
<td>4</td>
<td>1:800</td>
<td>Plate/cold GBBS</td>
<td>4068/090309</td>
<td>66</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>1:800</td>
<td>Plate/cold GBBS</td>
<td>4068/090309</td>
<td>64</td>
<td>0.57</td>
</tr>
<tr>
<td>6</td>
<td>1:500</td>
<td>Test tubes/cold GBBS</td>
<td>4068/090309</td>
<td>56</td>
<td>0.51</td>
</tr>
<tr>
<td>7</td>
<td>1:500</td>
<td>Test tubes/cold GBBS</td>
<td>4068/090309</td>
<td>57</td>
<td>0.66</td>
</tr>
<tr>
<td>8</td>
<td>1:800</td>
<td>Test tubes/cold GBBS</td>
<td>4068/090309</td>
<td>60</td>
<td>0.51</td>
</tr>
<tr>
<td>9</td>
<td>1:800</td>
<td>Test tubes/cold GBBS</td>
<td>4068/090309</td>
<td>54</td>
<td>0.54</td>
</tr>
<tr>
<td>10</td>
<td>1:500</td>
<td>Test tubes/cold GBBS</td>
<td>4068/090330</td>
<td>49</td>
<td>0.49</td>
</tr>
<tr>
<td>11</td>
<td>1:800</td>
<td>Test tubes/cold GBBS</td>
<td>4068/090330</td>
<td>59</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Table 9. Average results of ACA of the BRP pos. control and the In house control, comparing two haemolysin dilutions. The averages calculated are from table 8.

<table>
<thead>
<tr>
<th>Performance</th>
<th>Hemolysin dilution</th>
<th>Averages</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BRP pos. control (%)</td>
<td>In house control (CH50/mg IgG)</td>
<td></td>
</tr>
<tr>
<td>Plate/cold GBBS</td>
<td>1:500</td>
<td>57</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Plate/cold GBBS</td>
<td>1:800</td>
<td>65</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Test tubes/cold GBBS</td>
<td>1:500</td>
<td>54</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Test tubes/cold GBBS</td>
<td>1:800</td>
<td>58</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

5.2.8 Impact on ACA by Different Complement Activities

The In house control and the BRP pos. control show up different behaviour with increasing complement activity. This is when neither the method of choice nor the haemolysin dilution is considered. A higher complement activity results in a lower BRP pos. control, whereas a higher In house control result is achieved, see fig. 16 and 17. Hence, a lower complement activity seems to more easily result in an approved BRP pos. control, according to this data set. In theory, a higher complement activity should result in a higher ACA. Therefore is the result of the In house control desired and not that of the BRP. The data from the assays in table 10 are from the same assay occasions as in table 8 and are illustrated in fig. 16 and 17.

As mentioned, the haemolysin dilution and plate or tube method are not taken into consideration for the comparison below. However, all data is from the same assays so the graphs in fig. 16 and 17 should be comparable. This test should not be accredited with too
much significance though, due to the aforementioned reasons. The trend lines may behave differently comparing plate and test tube performance. This aspect could be considered in further investigations.

Table 10. Complement Activities and Corresponding Control Results.

<table>
<thead>
<tr>
<th>Complement activity (CH50/mL)</th>
<th>BRP positive (%)</th>
<th>In house control (CH50/mg IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.7</td>
<td>60</td>
<td>0.48</td>
</tr>
<tr>
<td>89.7</td>
<td>66</td>
<td>0.36</td>
</tr>
<tr>
<td>88.7</td>
<td>46</td>
<td>0.49</td>
</tr>
<tr>
<td>78.3</td>
<td>66</td>
<td>0.52</td>
</tr>
<tr>
<td>91.7</td>
<td>64</td>
<td>0.57</td>
</tr>
<tr>
<td>103</td>
<td>56</td>
<td>0.51</td>
</tr>
<tr>
<td>113</td>
<td>57</td>
<td>0.66</td>
</tr>
<tr>
<td>80.1</td>
<td>60</td>
<td>0.51</td>
</tr>
<tr>
<td>96.8</td>
<td>54</td>
<td>0.54</td>
</tr>
<tr>
<td>114</td>
<td>49</td>
<td>0.49</td>
</tr>
<tr>
<td>93.2</td>
<td>59</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Fig.16. Relation between the BRP Pos-Control and the Complement Activity.

Fig.17. Relation between the In House Control and the Complement Activity.
5.2.9 The Effect of Different Predilutions of the Complement for the BRP Pos. Control, Test Tube Performance

Results from a run showed that the haemolysis degree increases faster in the dilution series for the BRP pos. control when complement is prediluted 1:50 compared to predilution 1:100. The slope for predilution 1:100 is 0.28 and for 1:50 0.34. The graph for the dilution series of the BRP pos. control with the 1:50 dilution was rejected due to less than four approved dilution points between 15 and 85% haemolysis. The 1:100 predilution resulted in an approved graph. The result for predilution 1:50 is presented in fig. 18 and for predilution 1:100 is presented in fig. 19. Y in the figures represents the degree of haemolysis in %. Each point represents one test tube or well of one dilution series.

![Graph 18](image1.png)

Fig. 18. BRP pos. control result when complement is prediluted 1:50. The graph contains too few points between 15 and 85% haemolysis to be approved. Y is the degree of haemolysis in %.

![Graph 19](image2.png)

Fig. 19. BRP pos. control result when complement is prediluted 1:100. The BRP pos. control was 59%. Y is the degree of haemolysis in %.

5.2.10 Comparison of ACA Methods: Plate versus Tube Performance

When looking at the result from the two assays performed in plate and test tubes on the same day, all batches but two were slightly higher in ACA for plate than for test tubes. The In
house control and the BRP pos. control were also higher for plate than for test tubes. The assay performed with test tubes was not approved due to that the BRP pos. control was <60%.

The different results could be due to different laboratory technicians.

Table 11. ACA results for six batches of Octagam® 5%, comparing outcomes of the plate and the tube method when assays were performed the same day. ACA was higher for the plate method for four out of six batches. The ACA of the In house control and the BRP pos. control were also higher for plate than for test tubes.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACA (CH50/mg IgG)</td>
</tr>
<tr>
<td>1</td>
<td>0.47</td>
</tr>
<tr>
<td>2</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>0.79</td>
</tr>
<tr>
<td>5</td>
<td>0.61</td>
</tr>
<tr>
<td>6</td>
<td>0.72</td>
</tr>
<tr>
<td>In House Control</td>
<td>0.58</td>
</tr>
<tr>
<td>BRP pos. (%)</td>
<td>69</td>
</tr>
<tr>
<td>BRP neg. (%)</td>
<td>14</td>
</tr>
</tbody>
</table>

5.2.11 Different Sheep Blood Suppliers, Test Tube Performance

The BRP pos. control results for the four assay occasions are presented in table 12. The BRP pos. control was approved twice, being exactly 60%. In run 2, pooled blood from Siemens was used, whereas in run 3, non-pooled blood from SVA was used. It does not from these tests seem to be difference between different suppliers of blood. Next SVA batch may result in another ACA of the BRP pos. control as it has been seen that BRP pos. control results vary for different batches of blood (refer to chapter 5.1.1).

A tendency to get a lower BRP pos. control result for the second set of series is seen. This may be due to waiting time and temperature difference between the two series, giving different circumstances for the two sets of biological systems. For all steps, the first set of dilution series are treated before the second.

Table 12. Analysis Results Comparing Different Batches of Sheep Blood.

<table>
<thead>
<tr>
<th>Run</th>
<th>Blood batch</th>
<th>ACA Human BRP positive control (%)</th>
<th>Blood pooled? (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3030248</td>
<td>58 56</td>
<td>Y</td>
</tr>
<tr>
<td>2</td>
<td>3030248</td>
<td>60 50</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>4068/090803</td>
<td>60 52</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>3030249</td>
<td>54 49</td>
<td>Y</td>
</tr>
</tbody>
</table>
5.3 Final Discussion

ACA is a cumbersome method to deal with. As it is such sensitive to different method parameters, it is not actually a robust method. It is old and there should be other more modern techniques able to measure the same things that could replace the method if they only were developed and capable for doing that. Automation is one step in the right direction and should be aspired. For additional techniques, ELISA methods could be one approach as antibodies are involved in the assays. ELISA techniques have already been tested and compared to the reference method in the European Pharmacopoeia by Ramasamy et al.20 These tests showed that ACA depends both upon the physical characteristic and the concentration of aggregates. Therefore, methods applied for ACA should be precise and reliable. The ELISA had the advantage in having a higher precision and being less dependent on reagent source. Moreover, ELISA requires less technical expertise than the reference method in the European Pharmacopoeia. In the thesis study, the plate method was more precise than the test tube method. In combination with its simplicity, ELISA techniques would be preferable for determination of ACA.20 However, a long process of research, development, validation studies and regulatory involvement would be needed to get an ELISA technique approved for routine analysis of ACA at an FDA regulated plasma product plant.

The gelatine supplier and the automation aspect were not investigated in this thesis. When the Genesis RSP 150 robot is used for dilution steps, the GBBS is cooled, but no ice-bath can be used in the robot. If it is the additional cooling that is needed for reliable assays, it is not possible with the modified test tube method. The plate method is not utilizing cold GBBS but works anyway. What this depends on can be discussed. The incubated volumes are lower for the second incubation when the assay is performed in microtiter plate compared to test tube performance. Surface activation by the plastic in the plate wells may occur. Also, different incubators (air incubator vs. water bath) may have impact due to heat transfer differences. When plate is used, an additional transfer step in the robot has to be performed. It has earlier been discovered by Miekka et al.26 that ACA of IgG can be increased up to 20-fold by pipetting during the preparation of serial dilutions for the assay. The artificial increase can be diminished by addition of different stabilizers (for example gelatine, which is already used in the GBBS). The study by Miekka et al. opens up for further investigation of the impact on ACA by different pipetting steps in the assay.

If the additional transfer pipetting in the plate method overestimates the BRP pos. control as well as samples, the difference between the modified test tube method and the plate method can partly be explained. The plate method could possibly be used for ACA determination anyway as the ACA results will be falsely overestimated as well. It is not a danger to the patient though, since out of specification alarms (OOS) will be reported more easily with the plate method than with the test tube method. Actually, it could rather be a safety aspect to apply the plate method for determination of ACA in Octagam® batches.
6 Conclusions

Is the plate and the modified test tube method comparable?

The plate method results in 8% higher average of the BRP pos. control compared to the tube method. The tube method results in an average BRP pos. control below 60% (60-100% is the limits for approval) and is seldom approved. A better precision is gained with the plate method than the tube method. This in combination with approved BRP positive controls and the higher degree of automation makes the plate method advantageous in many aspects.

The plate method renders slightly higher ACA of Octagam® samples for four out of six batches than what is achieved with test tube performance. Further investigations are needed in order to reject or confirm that the method of choice is critical to the sample results.

The complement titer gets significantly higher when complement titration is performed on plate than when test tube performance is employed. This is irrelevant to the ACA outcome though, as the titer is adjusted for the ACA assay.

The two ACA methods are not giving similar BRP pos. control results and also different complement titers. They are therefore not comparable with regard to the BRP and the complement titration. The In house control is outside the scope of this comparison and has not been investigated. Therefore, no conclusions can be drawn regarding how Octagam® samples are affected by the two methods.

Which parameters in the method in general, influence on its robustness?

The SRBCs:

- The absorbance of SRBC + Milli-Q® changes by time and is sensitive to temperature variations.

- The complement titer and the ACA of the BRP pos. control vary for different batches of blood, especially for different sheeps.

- The complement titer decreases linearly to the age of the sheep blood.

- Non-pooled blood from sheep 4068 (batch 4068/090803) (SVA) results in similar ACA results as pooled blood from Siemens AB. ACA with the next batch of non-pooled blood may deviate from this batch though, since BRP pos. control results differ for different batches of non-pooled blood.

The more the haemolysin is diluted, the higher gets the BRP pos. control and the In house control. The difference for the BRP pos. control is the most obvious.

Increased complement activities affect the BRP pos. control and the In house control in opposite ways.

Consequently, the blood quality, the haemolysin dilution and the complement activity are critical parameters to the ACA method.
Which method that is used of plate and test tube is also critical. The difference between the methods may depend on many parameters; incubation circumstances, automation and temperature of the GBBS to mention a few.

**Is the BRP control adequate as a reference for the method in general?**

The Human BRP control is, according to this study, more adequate as a reference for the plate method than for the test tube method. The BRP pos. control is most often approved when the plate method is applied. The unsuitability for test tubes is due to that ACA of the BRP positive control often is lower than the limit for approval.

Furthermore, the In house control of Octagam® 5% and the BRP pos. control are not positively correlated with test tube performance according to this study. The adequacy of the BRP control is in the context of correlation with the In house control, higher with the plate method.

The In house control is believed to be representative as a reference since it is of the same nature as the samples analysed.

**Is the BRP positive control sensitive to different predilutions of the complement?**

Complement predilution 1:100 should be used rather than a 1:50 dilution in order to gain at least four dilution points between 15-85% haemolysis for the BRP positive control.
7 Recommendations for Further Investigation

The ACA method is influenced by a lot of parameters that vary from time to time, and the RSD for the method is 25%. Hence, a lot of tests for each parameter would be needed to confirm which parameters actually have the most impact on the robustness of the method. Suggestions for continued robustness testing follows:

- There may be an impact of robot transfer from test tubes to plate for incubation, influencing on the ACA for the plate performance. This factor should be studied.
- Time for cooling after the two incubations could be altered to see if a long cooling procedure on ice would impact on either of the methods.
- As the ACA assay itself is including antibodies, which have binding properties, it could be interesting to compare ACA results on the two plates with different binding abilities, tested for complement titration.

In order to increase the precision and reliability of the method, the following changes may help:

- Haemolysin titration should be performed for every new batch of complement and for every new batch of haemolysin, so the assay is not affected by a non-optimal diluted haemolysin.
- SRBC should be pooled from several sheep donors to reduce blood batch variations and possibly increase the precision of the method. The 5% SRBC solution should be newly prepared when it is used to avoid absorbance drifting.
- The complement for the BRP pos. control should be prediluted 1:100 instead of 1:50 as more dilution points are approved for the 1:100 dilution. The more points the line could be fitted to, the trustworthier is the result.
8 Acknowledgement

I would like to express my gratitude to all those who gave me the possibility to complete this thesis:

- Octapharma AB that came up with this thesis.
- Ann-Charlotte Hinz at Octapharma for being my supervisor, taking your time to discuss my experiments. Thank you for your encouragement and for valuable feedback on my report.
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- Carl-Fredrik Mandenius at Linköping University for being my examiner of this thesis.
- My opponent Elin Nyman. Thanks for feedback on my report.
- My boyfriend Johan Mårtenson. You have offered great support during the time for the thesis. Having you by my side is invaluable.
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- All of my friends. I am sorry that I have not had enough time for you when I have had two works to do. I am glad you had patience for that. Now it is time for a change!
- Finally: My family. Thank you for shown interest in my thesis and for always being there when I need you.

Written when summer turns to fall, a chilly afternoon in September 2009.

Ann-Louise Borg
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7 Introduction days for new employees 2009-04-15 – 2009-04-16 at Octapharma.


9 Internal material at Octapharma. ”Våra produkter och marknader”


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APPENDIX A  Statistics

Formulas used for paired two-sample tests of means and for Standard Deviation (SD) and Relative Standard Deviation (RSD).

Under the assumption that two samples have identical variances, the following formulae have been used to calculate the test statistic in two-sample tests of paired means:

\[ t_0 = \frac{\bar{y}_1 - \bar{y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \]

where \( \bar{y}_1 \) and \( \bar{y}_2 \) are the sample means, \( n_1 \) and \( n_2 \) are the sample sizes, \( S_p^2 \) is the estimate of the variance \( \sigma_1^2 = \sigma_2^2 = \sigma^2 \) is estimated from the formulae:

\[ S_p^2 = \frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2} \]

where \( S_1^2 \) and \( S_2^2 \) are the sample variances for the two samples.

If \( t_0 > t_{\alpha,n_1+n_2-2} \), where \( \alpha = 0.05 \), the null hypothesis have been rejected.

Standard Deviation (SD) for samples has been calculated according to the formulae:

\[ SD = \sqrt{\frac{1}{n-1} \sum_{j=1}^{n} (y_j - \bar{y})^2} \]

Relative Standard Deviation (RSD) for samples has been calculated according to the formulae:

\[ RSD = \frac{\sqrt{\frac{1}{n-1} \sum_{j=1}^{n} (y_j - \bar{y})^2}}{\bar{y}} \times 100 \]
APPENDIX B  
Data of absorbance experiments

The measured absorbances are from mixtures of 200µL 5% SRBC and 2.8mL Milli-Q®.

Absorbance measurement results for 200µL 5% SRBC + 2.8mL MilliQ H₂O, 0 days after opening. The mixture was kept in RT.

<table>
<thead>
<tr>
<th>Sheep ID 304, RT, 0 days after opening</th>
<th>Time after preparation (min)</th>
<th>Absorbance (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.6166</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.6291</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.6446</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.6465</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.5982</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>0.6240</td>
</tr>
</tbody>
</table>

Absorbance measurement results for 200µL 5% SRBC + 2.8mL MilliQ H₂O, 15 days after opening. The mixture was kept in RT.

<table>
<thead>
<tr>
<th>Sheep ID 304, RT 15 days after opening</th>
<th>Time after preparation (min)</th>
<th>Absorbance (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.6052</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.6342</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.6139</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.5984</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.6025</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>0.6108</td>
</tr>
</tbody>
</table>

Absorbance measurement results for 200µL 5% SRBC + 2.8mL MilliQ H₂O, 0 days after opening. The mixture was kept in 2-8°C.

<table>
<thead>
<tr>
<th>Sheep ID 304, 2-8°C 0 days after opening</th>
<th>Time after preparation (min)</th>
<th>Absorbance (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.6023</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.6536</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.6449</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.6782</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.6483</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>0.6717</td>
</tr>
</tbody>
</table>
Absorbance measurement results for 200µL 5% SRBC + 2.8mL MilliQ H₂O, 15 days after opening. The mixture was kept in 2-8°C.

<table>
<thead>
<tr>
<th>Sheep ID 304, 2-8°C</th>
<th>Time after preparation (min)</th>
<th>Absorbance (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 days after opening</td>
<td>0</td>
<td>0.6052</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.6374</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.6198</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.6374</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.6241</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>0.6019</td>
</tr>
</tbody>
</table>
Multiple linear regression of the variables used for prediction of the complement titer. The only variable significantly influencing on the complement titer in this model is the “Time in days since the blood container was opened” variable.

**APPENDIX C**

SPSS 17.0 output

The table below shows the variables entered and removed from the model, along with the model summary and ANOVA table.

### Variables Entered/Removed

<table>
<thead>
<tr>
<th>Model</th>
<th>Variables Entered</th>
<th>Variables Removed</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Time in days since the blood container was opened, Plate generating the titer, Plate used first in Genesis RSP 150</td>
<td>.</td>
<td>Enter</td>
</tr>
</tbody>
</table>

a. All requested variables entered.

### Model Summary

<table>
<thead>
<tr>
<th>Model</th>
<th>R</th>
<th>R Square</th>
<th>Adjusted R Square</th>
<th>Std. Error of the Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.847**</td>
<td>.718</td>
<td>.507</td>
<td>6.31392</td>
</tr>
</tbody>
</table>

a. Predictors: (Constant), Time in days since the blood container was opened, Plate generating the titer, Plate used first in Genesis RSP 150

### ANOVA

<table>
<thead>
<tr>
<th>Model</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Regression</td>
<td>406,389</td>
<td>3</td>
<td>135,463</td>
<td>3.399</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>159,436</td>
<td>4</td>
<td>39,859</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>565,825</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Predictors: (Constant), Time in days since the blood container was opened, Plate generating the titer, Plate used first in Genesis RSP 150
b. Dependent Variable: Complement titer in CH50/mL
<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>T</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(Constant)</td>
<td>290,409</td>
<td>5,668</td>
<td>51,233</td>
</tr>
<tr>
<td></td>
<td>Plate generating the titer</td>
<td>-1,734</td>
<td>4,464</td>
<td>-.103</td>
</tr>
<tr>
<td></td>
<td>Plate used first in Genesis</td>
<td>-1,973</td>
<td>4,643</td>
<td>-.117</td>
</tr>
<tr>
<td></td>
<td>RSP 150</td>
<td>-4,001</td>
<td>1,275</td>
<td>-.866</td>
</tr>
<tr>
<td></td>
<td>Time in days since the blood container was opened</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Dependent Variable: Complement titer in CH50/mL