Quantification of alpha-synuclein in cerebrospinal fluid

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**Abstract**

To date there is no accepted clinical diagnostic test for Parkinson’s disease (PD) based on biochemical analyses of blood or cerebrospinal fluid. Currently, diagnosis, measurement of disease progression and response to therapeutic intervention are based on clinical observation, but the first neuronal dysfunction precede the earliest recognition of symptom by at least 5 - 10 years. A potential diagnostic biomarker is oligomeric alpha-synuclein which in recent papers have reported a significant quantitative difference between PD and controls. In this master thesis, a method for measuring oligomeric levels of alpha-synuclein is presented together with a monomeric measuring commercial kit used to measure alpha-synuclein in a pre-clinical model of PD. A significant difference of monomeric levels could be detected between two weeks and four weeks post injection of a vector containing the gene for human alpha-synuclein, no significant difference between four and eight weeks was found.
Abstract

To date there is no accepted clinical diagnostic test for Parkinson’s disease (PD) based on biochemical analyses of blood or cerebrospinal fluid. Currently, diagnosis, measurement of disease progression and response to therapeutic intervention are based on clinical observation, but the first neuronal dysfunction precede the earliest recognition of symptom by at least 5 - 10 years. A potential diagnostic biomarker is oligomeric alpha-synuclein which in recent papers have reported a significant quantitative difference between PD and controls. In this master thesis, a method for measuring oligomeric levels of alpha-synuclein is presented together with a monomeric measuring commercial kit used to measure alpha-synuclein in a pre-clinical model of PD. A significant difference of monomeric levels could be detected between two weeks and four weeks post injection of a vector containing the gene for human alpha-synuclein, no significant difference between four and eight weeks was found.

Sammanfattning

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<th>Definition</th>
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<td>α-syn</td>
<td>alpha-synuclein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DoE</td>
<td>Design of Experiments</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy Bodies</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower Limit of Quantification</td>
</tr>
<tr>
<td>LN</td>
<td>Lewy Neuritis</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>NFDM</td>
<td>Non-fat Dry Milk</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>OFAT</td>
<td>One-factor-at-a-time</td>
</tr>
<tr>
<td>p-NPP</td>
<td>p-nitro-phenylphosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>ULOQ</td>
<td>Upper Limit of Quantification</td>
</tr>
<tr>
<td>rAAv</td>
<td>Recombinant adeno-associated viral vector</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia Nigra pars compacta</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’ tetramethylbenzidine</td>
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Chapter 1

Introduction

Currently there is no accepted clinical diagnostic test for Parkinson’s Disease (PD) based on biochemical analyses. To date, diagnosis is based on clinical observation which is highly unfortunate as the symptoms occur when a significant neurodegeneration have already occurred. alpha-synuclein (α-syn) is suggested to have a critical role in familial PD, based on the discovery of mutations in the SNCA gene which encodes α-syn and the following accumulation of α-syn in Lewy Bodies (LB). Consequently studies investigating quantitative differences of monomeric α-syn between PD and controls have been carried out, results from these have given inconclusive and contradictory data. More recent papers have reported on oligomeric (aggregated) α-syn in Cerebrospinal Fluid (CSF) indicating a significant quantitative difference between PD and controls. However, which forms, how, when and where they are involved in the contribution to PD remains elusive [4]. There is a critical need for both diagnostic and progression markers for PD. Oligomeric α-syn might be that biomarker but much research is still needed before a validated biomarker for PD is established and recognized. In addition, levels of monomeric and oligomeric α-syn might be used in pre-clinical and clinical studies evaluating the effects from compounds intended for disease modifying treatment in PD.

1.1 Aim

This project will focus on validating commercially available ELISA assays measuring monomeric α-syn according to AstraZeneca in house criteria. When validated, the ELISA assay will be used to evaluate a pre-clinical model of PD, levels of α-syn will be measured in rodents 2, 4, and 8 weeks post gene therapy treatment, respectively. The project will also include assay development of an ELISA measuring oligomeric α-syn in human and rat CSF. These assays will be used to evaluate the therapeutic effect after treatment in a pre-clinical model of PD. If CSF levels of α-syn are affected after treatment, these assays, facilitating the measurements of α-syn, will be included as exploratory biomarkers in a clinical trial.
1.2 AstraZeneca

The company AstraZeneca, was founded in 1999 through a merger of Swedish Astra, with its headquarters in Södertälje, Figure 1.1 and British Zeneca. With 61,000 employees divided in over 100 countries, sales exceeding 33 billion USD 2010 and with 10 drugs selling for 1 billion USD a year makes AstraZeneca one of the worlds leading pharmaceutical companies. AstraZeneca discovers, develops and market over-the-counter pharmaceuticals in six important areas of therapy: Cardiovascular, Gastrointestinal, Infection, Neuroscience, Oncology and Inflammation [5].

1.3 Parkinson’s Disease

PD is the second most common degenerative disorder following Alzheimer’s Disease, affecting about 1.6% of the general population older than 60-years [6, 7]. The first detailed description of PD was published by doctor James Parkinson in ”An Essay of the Shaking Palsy” in 1817. Patients with PD exhibit movement-related symptoms like shaking, rigidity, and slowness of movement. Later stages might include symptoms like cognitive and behavioural problems along with dementia. PD is a progressive degenerate illness which only manifest itself clinically after the pathology has reached an advanced stage [8, 9], these symptoms occur when approximately 50% of dopaminergic neurons projecting from the Substantia Nigra pars compacta (SNc) to the striatum, Figure 1.2, are lost [10].

1.3.1 Rodent Model of Parkinson’s Disease

This project involves the measurement of $\alpha$-syn in a pre-clinical model of PD. Rats will be treated with a Recombinant adeno-associated viral vector (rAAv) to express the human gene for $\alpha$-syn and terminated after 2, 4, and 8 weeks respectively.
Levels of monomeric and if possible, oligomeric \( \alpha \)-syn will be measured in order to evaluate the expression levels of the human gene.

## 1.4 \( \alpha \)-Synuclein

In patients with PD and other neurodegenerative disorders, deposits of \( \alpha \)-syn have been identified in aggregates such as LB and Lewy Neuritis (LN) which is the pathological hallmark of PD [11]. These seems to be not merely enclosed to the brain but also present in autonomic nerves in the heart, gut, and prostate [12–14]. The mechanisms that precede the irregularities of \( \alpha \)-syn remain poorly understood, however it has been shown that abnormal depositions of \( \alpha \)-syn occurs early in the disease process of PD and that following widespread accumulation is thought to, at least in part, be the cause of cognitive and behavioral deficits in PD with dementia [15].

\( \alpha \)-syn is a 14 kDa protein which is abundantly expressed in neuronal pre-synaptic terminals and have been suggested to play a role in vesicular transport [16], which could imply a role in synaptic dynamics though the entire physiological function of \( \alpha \)-syn remain to be explained. The link between expression levels of certain proteins and neurodegeneration is well established [3]. Detection of \( \alpha \)-syn in patients with synucleinopathies, such as PD, has yielded promising but inconclusive results [17]. In recent years it has been discussed that not the final aggregates nor the monomeric forms, but rather the oligomeric intermediates that might be the toxic species, see Figure 1.3 [3].

### 1.4.1 \( \alpha \)-Synuclein as a biomarker for PD

Currently, diagnosis, measurement of progression, and response to therapeutic intervention rely upon clinical observation [18] but the first neuronal dysfunction
Figure 1.3. Aggregate formation from monomeric α-syn, recent papers have been investigating whether it might be the oligomeric forms that are the toxic species. Size of different species are not to scale. Figure taken from [2].

Figure 1.4. Illustration showing neuronal degradation in patients with neurodegenerative disease. a, To date, diagnosis is based upon clinical observation which is possible only when the disease is already far advanced. In reality, the disease progression is probably altered over time and hence making it non-linear. b, If the disease could be discovered and consequently treated earlier, symptoms could be significantly postponed. Figure taken from [3].

precede the earliest recognition of symptoms by at least 5-10 years [3]. As PD is a progressive disorder, early diagnosis based on observation is particularly susceptible to error. Clinical observation is further obstructed by the ambiguous symptoms of PD, many of which overlap with symptoms of other diseases like progressive supranuclear palsy (PSP), multiple system atrophy (MSA), and corticobasal degeneration (CBD) each of which has distinct pathology, treatment, and prognosis [19]. Treatment optimization of PD requires accurate information about the ongoing disease [20]. Could the presymptomatic disease be diagnosed and treated prior to substantial neuronal loss, much would be won, Figure 1.4. To date, a definitive diagnosis can however only be established by analyzing autopsied brain tissue for confirmation of dopaminergic neural loss in the SNc and the presence of LB and LN [17].

Once diagnosis is established, another valuable role is to provide surrogate end-points. This could potentially enable a reduction in both the duration of a trial and the number of patients required for significance, as in the case with myocardial infarction and stroke where blood pressure and cholesterol have been
clearly linked to mortality in large trials and are now accepted end-points for drug licensing [20,21]. To date, there is no such biomarker for PD.

In one study by El-Agnaf et al. [22] oligomeric forms of α-syn in plasma was shown to be higher in PD compared to controls. Shi et al. [23] however, reported that more than 95% of α-syn resides in red blood cells and 1-4% in platelets, indicating both that minor variations in platelet contamination or hemolysis can have significant effects on measured α-syn levels in plasma. This is an indication on the importance of adequate control of blood contamination when analyzing samples, hence making α-syn in plasma hard to interpret. Less accessible but seemingly more robust indicator is CSF measurements of α-syn. There are several studies indicating lower levels in PD patients compared with controls [24–26]. Nonetheless, the mechanism of the decreased total levels of α-syn remains elusive. Various mechanisms are plausible, a reduction in release rate to extracellular space because of aggregation; enhanced clearance rate of α-syn from CSF; alterations in SNCA gene transcription [27]; mRNA splicing [28], yet unknown factors or a combination of these.
Chapter 2

Theory

2.1 Enzyme-Linked Immunosorbent Assay

*Enzyme-Linked Immunosorbent Assay* (ELISA) is a method which employs antibodies to detect a target antigen. An enzyme is covalently linked to a specific antibody that recognizes a target antigen as can be seen in Figure 2.1. If the antigen is present, the enzyme-antibody complex will bind to it, on the addition of substrate the enzyme will catalyze the reaction and produce a colored product. ELISA can be performed with either mono- or polyclonal antibodies and can detect antigen in the low picogram concentrations. Indirect ELISA is used to detect

![Figure 2.1. Schematic illustration of a sandwich ELISA on a 96 well plate; A antibody bound to the plate captures the analyte of interest which is then detected by the biotin labeled detection antibody. Visualization is here enabled by adding Streptavidin-HRP with high affinity for biotin.](image)
the presence of antibodies. The antigen is adsorbed to the bottom of a well and the sample to be analyzed is added, if the antibody of interest is present in the sample, it will bind to the antigen. Finally an enzyme-linked antibody which in turn recognizes the antibody in the sample is added (e.g. goat antibodies that recognize human antibodies) and unbound antibodies are washed away. Substrate is added, and if an enzyme reaction occurs it is implied that the sample contains antibodies against the antigen.

Sandwich ELISA is used to detect antigen rather than antibody. A capture antibody to a particular antigen is adsorbed to the well. Next the sample is added and finally the detection antibody, that is linked to an enzyme, and the analysis continues as in the indirect ELISA. In the Sandwich ELISA, the extent of the reaction carried out by the enzyme, is directly proportional to the amount of antigen present in the solution, hence this technique allows for the quantification of low concentrations [29].

2.1.1 Samples

The analyte is incubated with the polymer-bound antibody to form a complex. The concentration of analyte in the sample is proportional to the amount of antibody sites that bind analyte. A series of samples with known concentration of the analyte is applied to each plate to create a standard curve. The signal strength of unknown samples can then be compared with this standard curve and a relative concentration can be determined.

2.1.2 ELISA Coating

Through hydrophobic interaction between the antibody and the polystyrene plate, coating can be achieved with antibody concentrations of 1 - 10 µg/ml dissolved in Phosphate buffered saline (PBS) (pH 7.4) or carbonate-bicarbonate buffer (pH 9.4). Usually the plate is incubated for several hours to overnight at 4 - 37 °C. Optimal coating conditions can vary with each specific protein, some may require pretreatment or other conditions for optimal binding. Depending on the stability of the protein, coated plates can be used immediately or dried and stored at 4°C. In order to prevent nonspecific binding, especially of antibodies, coating should be performed using lower than the maximum binding capacity. As the concentration of antibody increases, the spacing between coated antibodies decreases, a phenomenon called "hooking". Hooking can occur when antibodies are trapped between bound antibodies. A result of hooking can be high background signal, as effective washing and removal of unbound proteins is prevented, which lowers the signal to noise ratio and hence the sensitivity of the assay is decreased. The Hook effect can also appear in assays where the analyte is present in concentrations higher than the capture and/or the detection antibody and hence become saturated. High concentrations of analyte could mimic far lower concentrations in the assay and lead to an estimation which is significantly lower than the true concentration. In order to avoid this effect, experiments with different dilutions must be carried out to establish the optimal concentrations of antibody and specimen.
2.1.3 Blocking- and Wash Buffers

When the microplate wells have been coated, the remaining surface area must be blocked to prevent unwanted binding to the plate in subsequent steps. As with the coating procedure, no blocking reagent or method is ideal for all assays, advantages and disadvantages and how these affect the assay must be considered. Non-ionic detergents, such as Tween 20, are one of the major classes of blocking reagents. Detergents provide a barrier to biomolecule attachment to the polystyrene plate which can be removed by washing with water or aqueous buffer and is thus referred to as temporary blockers. Detergents are preferably included in the wash buffer and not used solely as a blocking reagent [30]. Protein blockers can both permanently block non-occupied sites on the surface and stabilize biomolecules already bound to the surface to reduce steric hindrance and denaturation problems. Commonly used blockers are Bovine Serum Albumin (BSA) and Non-fat Dry Milk (NFDM), both are inexpensive but due to the mixture of proteins, some crossreactivity problems can occur [31], there are however also pure synthetic blockers available if crossreactivity issues occur.

Removal of unbound antigen or antibody is performed via a series of washes, a step where wells are consecutively filled with washing buffer and emptied, a balance between leaving too much unbound protein and stripping of specifically bound protein. In the washing step, optimization in regard to removal of unbound antigen or antibody is crucial for maximized assay sensitivity. One major factor that is to be considered in this category is composition of wash solution. Preferably, the wash solution consists of an enzyme friendly buffer with an addition of a detergent, such as PBS with 0.01-0.1% Tween 20, as detergents may aid in the removal of loosely bound protein with concurrent temporary blocking. To minimize background, instead of increasing the vigorousness of the wash buffer which could result in stripping of specifically bound protein, the optimal number (usually 3-5) of subsequent wash cycles should be determined, since washing is a dilution process of unwanted species.

2.1.4 Selecting Enzyme

Two of the most commonly used enzymes for ELISA are Horse Radish Peroxidase (HRP) and alkaline phosphatase, the popularity of these are based on their ability to produce a sensitive and inexpensive assay [32]. Both HRP and alkaline phosphatase are relatively stable, inexpensive and commercially available. Due to the small size of HRP, MW 40,000 g/mol, it rarely causes steric hindrance, which is not the case with alkaline phosphatase with MW 86,000 g/mol. Sodium azide is commonly used in biological buffers to reduce microbial contamination which, even in low concentrations, inactivates the activity of HRP [33]. Alkaline phosphatase is inactivated by acidic pH, chelating agents, and inorganic phosphates, hence PBS buffer cannot be used in the assay where it might come in contact with the enzyme wherever activity is desirable.

For HRP, the most widely used substrate is 3,3',5,5' tetramethylbenzidine (TMB) which produces a blue color when tetramethylbenzidine is oxidized to tetramethylbenzidine diimine measurable absorbance at 650 n. The reaction can
be stopped with 2 M H$_2$SO$_4$, resulting in a yellow color and read at 450 nm. With alkaline phosphatase, the substrate commonly used is *p*-nitro-phenylphosphate (p-NPP) which produces a yellow color measurable at 405-410 nm, the reaction can be stopped by adding 1 M NaOH \[32\].

### 2.1.5 Biotin

Biotin is a water-soluble, naturally occurring, vitamin and is necessary for cell growth, synthesis of fatty acids as well as the metabolism of amino acids and fats. Biotin is often linked to an antibody in biochemical assays, as in ELISA. The relatively small size of the biotin molecule, MW 244 g/mol enables the biological activity of the protein to, most likely, remain unaffected. *N*-Hydroxysuccinimide (NHS) esters are commonly used as biotinylation reagents which react efficiently with primary amino groups by nucleophilic attack, forming an amide bond and releasing the NHS \[34\]. Antibodies usually have several sites for labeling which in turn can amplify the signal, thereby increasing the sensitivity of the assay. The size and distribution of amino groups on the protein along with the amount of reagent used, determine the extent of biotin labeling.

### 2.2 Western Blot

Western blotting, also called immunoblotting as an antibody is used to detect its antigen, enables the possibility to identify a target protein in the midst of a complex protein mixture \[35\]. The first step is to separate the macromolecules using gel electrophoresis which may be by isoelectric point, molecular weight, electric charge or a combination of these factors. The gel consists of cross linked polymer matrix used to support and separate the molecules. By varying the density of the gel, optimized separation in a certain attribute range can be achieved.

Samples are loaded in small wells in the gel prior to applying the current, which in turn allows the molecules to migrate through the matrix. By utilizing a molecular weight marker which produces bands of known size, proteins of interest can be identified. The second step, which is performed to make the proteins accessible to antibody detection, is to transfer the separated proteins to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane which is placed on top of the gel. Basically the same principle as in the first step is used, the current is however applied 90 degrees to the gel. Once the proteins are bound to the membrane blocking is performed to prevent (or reduce) interaction between the membrane and the primary antibody used for detection, this is achieved by placing the membrane in a solution of BSA, NFDM or other blocking solution. Thus, the protein in the blocking solution attaches to the membrane in all places where the target protein has not. The blocked membrane is incubated in a solution containing the primary antibody which will bind to the target protein.

The secondary antibody, directed at a species-specific portion of the primary antibody, is added after rinsing the membrane and is typically linked to biotin or to a reporter enzyme such as alkaline phosphatase or HRP which will allow for visual identification. Enzyme substrate is applied after the unbound secondary
antibody is washed away, bands corresponding to the detected target protein will appear as dark regions on the developed film.

2.3 Design of Experiments

An effective way of finding solutions to complex problems is the approach offered by Design of Experiments (DoE) which allows to draw conclusions about the significant behavior of the studied experiment. Considering costs and time required for certain experiments, there are often limitations in the possible number of performed experiments. With DoE the number of experiments is kept as low as possible and the most informative combination of factors is chosen [36].

2.3.1 D-Optimal Design

A D-optimal design is generated by a computer algorithm and is suitable when there is a limitation in the number of experiments able to be carried out, the usefulness of D-optimal design as the number of factors increases can be seen in Table 2.1. The difference between a full factorial design and the D-optimal design can be illustrated with a three factor experiment as in Figure 2.2.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Full Factorial</th>
<th>Fractional Factorial</th>
<th>D-Optimal</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>32</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>32</td>
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<td>128</td>
<td>64</td>
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<td>256</td>
<td>64</td>
<td>43</td>
</tr>
<tr>
<td>9</td>
<td>512</td>
<td>128</td>
<td>52</td>
</tr>
</tbody>
</table>

The D-optimal design contains the best subset of all possible experiments which is created by a selection process [37]. First a candidate set matrix, containing all theoretically possible experiments, is constructed. Each row represents an experiment and each column a factor. Subsequently the design matrix $X$ is created which is an $n \times p$ matrix where the number of rows $n$ represents the number of experiments in the design and $p$ is the factors, including interactions. The number of runs included in the experiment can be chosen by the experimenter, but the lowest possible number of runs is equal to the number of factors, $p$, included in the model.

The selection of which experiments to include in the design matrix is dependent on a special criterion. The so called information matrix is constructed by transposing the design matrix $X$ and multiplying it with the original matrix, $(X^T X)$. The D-optimality method seeks to maximize the determinant of the information matrix by exchanging which columns to include, a tedious process always performed by a
Figure 2.2. Illustration of an experiment with three factors, A, B and C with 4, 3 and 2 levels respectively and the difference between a full factorial experiment, which would investigate all circles, and dark circles which would be chosen by the D-optimal algorithm. Hence the full factor would require all 4x3x2 points and the D-optimal experiment would reduce the number of experiments to 12.

computer as the number of calculations increase rapidly with increasing number of factors. This means that the optimal design matrix $X^*$ contains the $n$ experiments which maximizes the determinant of $(X^TX)$ and hence the information given by the experiments [38].
Chapter 3

System and process

In the very start of this master thesis, a rough plan was established to provide a general idea of available time. A Gantt chart providing a schematic overview can be seen in Figure 3.1. The project was divided in three major divisions to establish boundaries of time available for each part. Three interim goals was established, Interim goal 1: is the selection of antibody to be used in the novel oligomeric ELISA assay and Interim goal 2: is the selection of which monomeric commercial ELISA kit to be used and finally, Interim goal 3: characterizing the PD model. These goals were planned to be met by 2012-04-02, 2012-04-09, and 2012-05-07 respectively.

Figure 3.1. Gantt chart illustrating available and planned time for each part of the project.
3.0.2 First third of the project

First few weeks will be devoted to gather relevant information and practicing on the ELISA procedure to produce consistent results. When adequate information has been gathered a project plan will be established to provide an overview of the time available for each part of the project. In this third, the introduction and as much of the theory as possible, will be written.

3.0.3 Midway

Of the two parts which this master thesis consist, development of a novel oligomeric ELISA assay and validation of commercial ELISA kits, the first is considered the most problematic and obscure and was thus decided to be dealt with first.

Following weeks will be used for researching which methods and materials previously used for measuring oligomeric $\alpha$-syn and simultaneously carrying out experiments to verify these results. When an antibody and procedure have been decided upon, emphasis can put into optimizing and analyzing the assay where the antibody is to be used. Evaluation of commercial ELISA kits can be performed when the range of products available have been scanned and ordered.

3.0.4 Final part

Performing an ELISA measurement is a quite rapid process and hence the time planned for this is relatively short, some effort can probably be put into validation of the optimization. When the commercial ELISA kit is validated, evaluations can be performed on the pre-clinical model of PD. When the novel ELISA assay is optimized and validated it can be used for measurement of oligomeric $\alpha$-syn. Results and conclusions can then be put together in the final report which is planned to be finished by the end of may.

3.1 Plan for systematic follow-up

Interim goal: 1 is considered met when an antibody with sufficient sensitivity and specificity for $\alpha$-syn have been found and evaluated.

Interim goal: 2 is considered met when the chosen commercial ELISA kits for monomeric $\alpha$-syn have been validated according to AstraZeneca in house criteria for ELISA kits and approved for monomeric $\alpha$-syn measurements in the pre-clinical model.

Interim goal: 3 is considered met when the pre-clinical model of PD have been characterized and data have been analyzed.
Chapter 4

Criteria in ELISA validation

This project involved validation of commercially available ELISA assays measuring monomeric α-syn. These assays were validated according to AstraZeneca in house criteria.

4.0.1 Calibration curve performance

The calibration curve acceptance is based on a 5:7:20 rule. 5 of the back-calculated results for the 7 positive calibrations standards should be within 20% of their target concentration except for the Lower Limit of Quantification (LLOQ) and the Upper Limit of Quantification (ULOQ) which should be within 25%. If a calibration standard replicate have precision which exceeds 25%, it may be removed from the standard curve. LLOQ is determined for each plate, this is based on the back calculated concentration which has to be within 25% of the target concentration and the precision has to be <25%.

4.0.2 Assessment of drift

Drift is assessed by running duplicate samples on either side of the plate and comparing the back-calculated responses.

4.0.3 Inter-assay accuracy and precision

Inter-assay accuracy and precision is evaluated by running samples on different plates on different occasions.

4.0.4 Dilution linearity

The dilution linearity is determined by assaying different samples at different dilutions. This information is crucial as it provides information about the precision of assay results for samples tested at different dilutions. Linearity is relative to the calculated amount of analyte and is based on the standard curve, which usually is
not linear. Good linearity over a wide range of dilutions enables the possibility to dilute samples to ensure they fall within the standard curve or last for more tests.

4.0.5 Matrix effect

A spike recovery and linearity of dilution experiment is performed to validate the accuracy of the assay. Spike recovery is used to establish whether the result is influenced by the constituents of the sample. In the sample, the target analyte can be masked by the complex mixture in many biological samples. A known concentration of analyte is added (spiked) to the sample and to the diluent buffer. If the recovery from these are identical, there is no interference between the sample components and the analyte detection. Usually spike recovery is tested for several dilutions. Samples should be spiked in such manner that the dilution effect is small e.g <5%.

4.0.6 Cross reactivity

Cross reactivity measurements are performed using relevant variants of the analyte, e.g. β-synuclein and x-synuclein, for cross reactivity test for a α-syn specific mAb.

4.0.7 Hook effect

The Hook effect can lead to false negative determinations and usually appear when the concentration of analyte is larger than the concentration of assay antibodies and hence the antibodies becomes saturated. This will lead to significant underestimation of the true concentration in the assay [39].

4.1 Commercial ELISA kits

Three kits were evaluated in this project, Table 4.1.

Table 4.1. List of the three commercial ELISA kits chosen for the initial round of evaluation.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Catalog number</th>
<th>Specificity</th>
<th>Claimed LLOQ</th>
<th>Sample Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invitrogen</td>
<td>KHB0061</td>
<td>Human</td>
<td>230 pg/mL</td>
<td>50 μL</td>
</tr>
<tr>
<td>Covance</td>
<td>SIG-38974</td>
<td>Human</td>
<td>6.1 pg/mL</td>
<td>200 μL</td>
</tr>
<tr>
<td>Anaspec</td>
<td>55550</td>
<td>Human/Mouse/Rat</td>
<td>7.8 pg/mL</td>
<td>100 μL</td>
</tr>
</tbody>
</table>

An advantage of the Covance kit is the specificity for human α-syn as a rat preclinical model is used for a human expression of α-syn in the SNc. Evaluation of the model includes measurement of the human gene expression which only would be possible with a kit specific for human α-syn or a rat specific in combination with a human and rat measuring kit where the natural occurring levels could be
subtracted. A major drawback with the Covance kit is that it requires a massive 200 µL sample per well, which is problematic as the volume of CSF and plasma from the pre-clinical model is very limited. Half of the sample volume is required for the Anaspec kit, however it has no specificity for human α-syn which is not advantageous as the concentrations of the natural occurring rat α-syn are not known and hence cannot be subtracted from any measurements.
Chapter 5

Materials and Methods

5.1 Custom peptides and Antibodies

Two customized peptides (Bachem, Weil am Rhein, Germany) with different length along with full length monomeric α-syn were ordered to be used as the standard curve for measurement of oligomeric α-syn. The peptides consisted of a dimer with cystein bridges, both containing the sequence matching the epitope of antibodies used. Two antibodies were used in this project to detect α-syn oligomers. Syn211 mAb (Abcam, Cambridge, England) which recognizes amino acid 121-125 of human α-syn. LB509 (Abcam, Cambridge, England) a mouse monoclonal antibody to α-syn with epitope in amino acid 115-122. Both mAb are, according to the manufacturer, specific for α-syn and does not react with β-synuclein or γ-synuclein.

5.2 Western Blotting procedure

Samples were heated in 90°C for 5 min prior to gel loading. Samples and molecular weight marker SeeBlue Plus2 (Invitrogen, Carlsbad, CA) were loaded to NuPAGE 12% Bis-Tris Gel (Invitrogen) with MES SDS Running Buffer (Invitrogen) and run for 25 min at 200V. Blotting was then performed using iBlot Gel Transfer Stack (Invitrogen) for 7 min followed by 1 h of blocking in 5% NFDM in PBST 0.05% (PBS containing 0.05% Tween 20). Washing was performed in PBST 0.05% for 1 h while shaking, wash buffer was exchanged every 10 min. Incubation with primary mAb was performed O/N in 4-8°C followed by washing for 1 h. Secondary antibody, diluted to 100 ng/mL in 5% NFDM in PBST 0.05%, was added and incubated for 2 h followed by washing for 1 h and finally film was developed.

5.3 An ELISA to measure α-syn oligomers

The novel ELISA was based on a conventional sandwich system with capture of α-syn by a mAb and with detection using a biotinylated form of the same
mAb. Consecutively, the biotinylated mAb is detected by the Streptavidin-HRP complex followed by colometric enzyme substrate. When the capturing antibody in the ELISA assay bind the target protein, the epitope of that target will become occupied. So by utilizing the same mAb for both capturing and detecting, only non monomeric species is detected, Figure 5.1.

5.3.1 Preparation of coating buffer

A 0.05M NaHCO buffer, pH 9.6, was prepared by diluting 0.795 g NaCO and 1.465 g NaHCO$_3$ in 500 mL of MQ-water and stored at 4-8 °C for later use.

5.3.2 Preparation of the biotinylated antibody

EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh™ Format (Pierce, Rockford, IL, USA) was dissolved in ice-cold Milli-Q water to a final concentration of 1 mg/mL. 22.5 µL of this mixture was added to 500 µL (1 mg/mL) mAb LB509 (Abcam, Cambridge, England), gently vortexed and incubated at RT for 1 hour. Unbound biotinylated reagent was removed by adding the mixture to a Protein Desalting Spin Column with 7K cutoff (Thermo Scientific) and centrifuged at 1500 × g for two minutes.

5.3.3 Spike Recovery

Assessment of spike recovery was used by adding concentrated analyte, <5% of the total volume, to samples and dilution buffer in duplicate.

5.3.4 Cross reactivity

To evaluate possible cross reactivity with β-synuclein and α-synuclein a ELISA experiment was performed, evaluating different concentrations and corresponding cross reactivity.
5.3 An ELISA to measure α-syn oligomers

Table 5.1. List of the factors used in the D-optimal design and the three levels used for each.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary mAb dilution</td>
<td>-1 0 1</td>
</tr>
<tr>
<td>Secondary mAb dilution</td>
<td>1/500 1/1000 1/2000</td>
</tr>
<tr>
<td>Sample incubation time</td>
<td>0.5h 1h 2h</td>
</tr>
<tr>
<td>Secondary mAb incubation time</td>
<td>0.5h 1h 2h</td>
</tr>
<tr>
<td>Streptavidin-HRP dilution</td>
<td>1/100 1/200 1/400</td>
</tr>
</tbody>
</table>

5.3.5 General procedure for oligomeric measurements

An ELISA plate was coated by overnight incubation in 4-8°C with 1 µg/mL of monoclonal antibody (mAb) (100 uL/well), in NaHCO₃ buffer (pH 9.6), washed 5 times with PBST and incubated with 100 µL of the samples. Dilutions, if any, were carried out in 0.25% BSA in PBST. The plate was incubated at room temperature for 3 h with subsequent washing 5 times with PBST, 100 µL of biotinylated mAb diluted to 4 µg/mL in 0.25% BSA in PBST was added to each well and incubated at room temperature for 1 h. Wells were washed 5 times with PBST and incubated with 100 µL Streptavidin-HRP complex diluted 1/200 in 0.25% BSA in PBST for 30 minutes followed by 5 times washing with PBST. 100 µL/well of enzyme substrate TMB was then added and incubated in RT in the dark for 30 minutes. Absorbance values at 450 nm were determined using SpectraMax 340PC (Molecular Devices, SunnyVale, CA).

5.3.6 Optimizing Oligomeric Assay in regard to Signal to Noise and total time of analysis

Due to limitation in available time and resources, a full factor experiment was not possible to perform. Instead a D-optimal design was proposed using the statistical software Umetrics MODDE to find the optimal conditions under certain limitations, see Section 2.3.1. Based on experience, three levels for each of the five factors were chosen, and the optimal levels were evaluated. The levels for each of the five factors are summarized in Table 5.1.

A total of 31 experiments were performed with CSF diluted 1:2 in dilution buffer containing 0.25% BSA in PBS-T. All experiments were carried out in duplicate and the mean value of both signal and noise were calculated for each experiment. The three experiments with the highest Signal to Noise ratio can be seen in Table 6.1. To test all possible combinations of factors and levels, 3⁵ = 243 experiments would have to be carried out. Since only 31 actual experiments were conducted there was only a small possibility that the actual optimal experiment was performed. To find the optimal conditions, results from all 31 experiments were fitted to a model using multiple linear regression and the Signal to Noise ratio was optimized with a Nelder Mead simplex method aimed to maximize the ratio.
Chapter 6

Results and Discussion

In this project, a novel ELISA assay measuring oligomeric forms of α-syn was developed and a commercial ELISA kit was validated for the measurement of α-syn levels in a pre-clinical model of PD. The feasibility of the oligomeric assay was first evaluated and followed with optimization of the method. Monomeric measuring commercial kits were validated according to AstraZeneca in house criteria and followed by the measurement of human α-syn in rat CSF. These rats were terminated 2, 4, and 8 weeks post gene therapy respectively.

6.1 Oligomeric ELISA assay

mAb Syn211 displayed little to no affinity for α-syn. To test the feasibility of oligomeric ELISA assay was performed with mAb Syn211. No signal could be detected in human CSF, plasma, custom peptides, nor in high concentrations of synthetic full length α-syn. Western blotting was performed, both with a biotinylated Syn211 and an anti-α-syn mAb. High concentrations of monomeric α-syn (1000ng/ml) was detected but the method was considered too insensitive, further research was not conducted, data not shown. As mAb Syn211 failed to deliver in terms of sensitivity, another mAb was evaluated, LB509. Using the same test of feasibility, ELISA verified that mAb LB509 was fit for use in the oligomeric assay and demonstrate sensitivity in the lower ng/ml range of full length α-syn. The custom peptides, intended to be used as standard curve in the oligomeric assay, yielded no signal at any concentrations. ELISA experiments were carried out to establish sensitivity, cross reactivity, spike recovery and intra assay precision.

6.1.1 Optimization

As the total time of analysis in the general ELISA procedure, explained above, was quite long and no signal could be detected for the custom peptides, optimization was performed to determine whether the Signal to Noise ratio, total time of analysis, and sensitivity could be improved. Usually an One-factor-at-a-time (OFAT) method is employed in optimization of ELISA assays but as this method can not
Table 6.1. The three experiments of the total 31, which resulted in the highest ratio between Signal and Noise. (*) The calculated optimal levels of each factor, using the Nelder Mead simplex method.

<table>
<thead>
<tr>
<th>Signal to Noise</th>
<th>Primary mAb dilution</th>
<th>Secondary mAb dilution</th>
<th>Sample incubation time</th>
<th>Secondary mAb incubation time</th>
<th>Streptavidin-HRP dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.09</td>
<td>1/2000</td>
<td>1/500</td>
<td>2h</td>
<td>2h</td>
<td>1/100</td>
</tr>
<tr>
<td>7.14</td>
<td>1/2000</td>
<td>1/100</td>
<td>2h</td>
<td>2h</td>
<td>1/400</td>
</tr>
<tr>
<td>6.45</td>
<td>1/2000</td>
<td>1/500</td>
<td>2h</td>
<td>2h</td>
<td>1/400</td>
</tr>
<tr>
<td>8.2102*</td>
<td>1/2000</td>
<td>1/323</td>
<td>2h</td>
<td>2h</td>
<td>1/121</td>
</tr>
</tbody>
</table>

Table 6.2. Results of the estimated optimal levels of the five factors with previously used buffer (1) and fresh (2).

<table>
<thead>
<tr>
<th>Signal to Noise</th>
<th>Primary mAb dilution</th>
<th>Secondary mAb dilution</th>
<th>Sample incubation time</th>
<th>Secondary mAb incubation time</th>
<th>Streptavidin-HRP dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 1.36</td>
<td>1/2000</td>
<td>1/323</td>
<td>2h</td>
<td>2h</td>
<td>1/121</td>
</tr>
<tr>
<td>(2) 1.54</td>
<td>1/2000</td>
<td>1/323</td>
<td>2h</td>
<td>2h</td>
<td>1/121</td>
</tr>
</tbody>
</table>

estimate interactions another method was performed, D-optimal design. With D-optimal design, 31 experiments were constructed and performed and the three with highest Signal to Noise ratio can be seen in Table 6.1 together with the estimated optimal levels.

As the estimated optimal conditions had not been previously executed in any of the initial experiments, it was evaluated in a subsequent run. Experiments were duplicated and the difference between freshly made dilution buffer and the previously used was compared to resolve whether this could have biased the Signal to Noise ratio, results can be seen in 6.2.

The average noise in the optimization run was unexpectedly high, possibly due to protein degradation in the dilution buffer. In the subsequent validation run of the estimated optimals, experiments were run i duplicate with old, 4 days in 4-8°C, and fresh buffer respectively. As can be seen in Table 6.2, the old dilution buffer resulted in a higher noise, hence a lower Signal to Noise ratio. With the freshly prepared buffer noise was still an issue. To address this problem, the addition of another washing step, to a total of 5 washes between each step, should be considered. The high Signal to Noise ratio achieved in the optimization experiments could not be reproduced. More experiments is needed in order to make any conclusions. However as indicated in the optimization run, the global optimum is probably not within the ranges selected for the incubation times. However, a signal was detected from both types of custom peptides which had not been previously accomplished with the general ELISA procedure, see Section 5.1. This will provide a reliable standard curve in future measurements, using this
6.2 Evaluation of commercially available ELISA kits for detection of \( \alpha \)-syn

<table>
<thead>
<tr>
<th>Concentration (pg/mL) at beginning of plate</th>
<th>Concentration (pg/mL) at end of plate</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1136.6</td>
<td>917.7</td>
<td>81%</td>
</tr>
<tr>
<td>1298.2</td>
<td>1314.0</td>
<td>101%</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>91%</td>
</tr>
</tbody>
</table>

Table 6.3. Assessment of drift across two plates and the calculated accuracy using quality control samples with 1000 pg/ml of \( \alpha \)-syn.

Due to the limitations in sample size in the pre-clinical model of PD, measurements of oligomeric forms of \( \alpha \)-syn was not conducted. The reason for this was that measurements of monomeric levels was prioritized as no evaluation of this model had been performed before. However, the oligomeric model remains validated and is available for future use at AstraZeneca.

6.2 Evaluation of commercially available ELISA kits for detection of \( \alpha \)-syn

Three commercial ELISA kits for the measurement of monomeric \( \alpha \)-syn were evaluated as can be seen if Table 4.1. The Invitrogen kit was rapidly expelled as no signal could be observed in relevant concentrations of either CSF nor plasma, data not shown.

As the Anaspec kit was not specific for human \( \alpha \)-syn and no known levels of rat \( \alpha \)-syn was available and hence could not be subtracted from any measurements. Validation and measurements were performed using the ELISA kit from Covance.

6.2.1 Assessment of drift

Drift across a 96 well plate was assessed by running duplicate QC samples at either end of the plate and comparing the back-calculated responses.

The mean accuracy of the QC 25, QC 500, and QC 1000 samples analyzed at the end of the two different ELISA plates was 81% and 101% respectively, Table 6.3. A potential risk with drift should be considered when planning the analysis-order of studies. Drift should be monitored in future studies through QC performance at the start and end of the samples on each plate.

6.2.2 Inter-assay accuracy and precision

The inter-assay accuracy and precision data for QC samples was calculated from the analysis of each quality control sample in duplicate, on two separate occasions.

The inter-assay accuracy for the determination of QC 1000 and QC 25 was 108% and 113% respectively. The inter-assay precision was 5.35 and 7.2 respectively, Table 6.4.
Table 6.4. Inter-Assay data for the determination of α-syn in quality control samples.

<table>
<thead>
<tr>
<th>Date</th>
<th>QC Found concentration</th>
<th>QC Found concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>20120516</td>
<td>1032.7</td>
<td>27.3</td>
</tr>
<tr>
<td>20120525</td>
<td>1135.6</td>
<td>29.0</td>
</tr>
</tbody>
</table>

Mean accuracy 108% 113%

CV 5.35% 7.2%

Table 6.5. Low spike recovery of synthetic α-syn in Human CSF.

<table>
<thead>
<tr>
<th>Individual</th>
<th>No Spike</th>
<th>Low Spike (+250 pg/mL)</th>
<th>Expected</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0</td>
<td>139.7</td>
<td>250</td>
<td>56%</td>
</tr>
<tr>
<td>CSF Pool 1</td>
<td>806.9</td>
<td>1136.3</td>
<td>1056.9</td>
<td>108%</td>
</tr>
<tr>
<td>CSF Pool 2</td>
<td>825.7</td>
<td>990.5</td>
<td>1075.7</td>
<td>92%</td>
</tr>
</tbody>
</table>

Mean recovery 85%

6.2.3 Matrix Effect

Human CSF samples from were analyzed to assess potential interference from endogenous matrix components. Human CSF samples were analyzed unspiked and spiked at 250, and 600pg/ml using synthetic α-syn. The CSF samples were diluted 2-fold before analysis. No matrix effect could be seen in the samples analyzed, Table 6.5 and 6.6.

6.2.4 Quantification of monomeric α-syn in the pre-clinical model of PD

As no information was available regarding the expression levels of human α-syn levels, a couple of samples were analyzed to determine whether samples could

Table 6.6. High spike recovery of synthetic α-syn in Human CSF.

<table>
<thead>
<tr>
<th>Individual</th>
<th>No Spike</th>
<th>High Spike (+600 pg/mL)</th>
<th>Expected</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0</td>
<td>634.3</td>
<td>600</td>
<td>104%</td>
</tr>
<tr>
<td>CSF Pool 1</td>
<td>806.9</td>
<td>1392.9</td>
<td>1406.9</td>
<td>99%</td>
</tr>
<tr>
<td>CSF Pool 2</td>
<td>825.7</td>
<td>1422.6</td>
<td>1425.6</td>
<td>100%</td>
</tr>
</tbody>
</table>

Mean recovery 101%
be diluted, which could solve the issue with the large sample volumes required. Samples were analyze in 2- and 8-fold dilutions. Due to low sample volumes of CSF, the 1:2 dilution sample could only be analyzed in singlicate and hence no statistical confidence is given, however a small hint to the true levels is provided. As expected, the back calculated α-syn levels was higher in the 8 week rat than in the 2 week, ranging from 2172-3000 pg/mL to 452 pg/mL respectively, no signal could be detected for the 1:50 dilution for the 2 week rat. No signal could be detected in any dilutions of plasma. The highest point in the standard curve is 1500 pg/mL and the lowest is 6.1 pg/mL, if the samples analyzed are representative for the age groups, all samples could be diluted 1:8 and still fall within the range of the standard curve.

Measured levels in the pre-clinical model of PD can be seen in Figure 6.1. A significant difference can be seen between 2 and 4 week rats but no significant difference could be seen between 4 and 8 weeks. Levels of α-syn was believed to be higher 8 weeks after the rAAv had been injected. This provides information about the model which can be used in subsequent studies. The same model will be used in a project where rats will be treated with a MPO inhibitor in order to prevent neurodegeneration primarily in the SNc and striatum. Measurements of oligomeric levels of α-syn could not be conducted due to limitations in sample size, the method developed will however be used in future work at AstraZeneca as the MPO project will continue.
Chapter 7

Follow up on System and Process

As planned, first few weeks were devoted to researching the chosen method before the development of the oligomeric assay was commenced. An antibody previously used in a similar method, by a different research group, was ordered. As no signal could be detected in any ELISA experiment or Western blot, the question was raised, should another batch of the same antibody be ordered or should another be evaluated? Considering the limitations in time available for each part of this project, the latter was chosen as the risk of getting another antibody, not generating any signal, could not be accepted. Ordering a different antibody was thought to be safer in terms of generating a signal compared to ordering the same antibody again, even if from a different batch. As further experiments with the oligomeric assay had to be postponed until the new antibody had been delivered, evaluations of the commercial monomeric α-syn kits could be commenced. Three available kits were found and ordered. One of these was rapidly expelled as the sensitivity proved to be too low. The kit from Covance and Anaspec had similar sensitivity, but the latter was not specific for human α-syn which was undesirable. A few samples were analyzed from the pre-clinical model to see whether these could be diluted and still fall within the standard curve or not. If so, the kit from Anaspec could be expelled and the extra time spent on evaluating the first antibody in the oligomeric assay could be regained. As the samples could be diluted, no time had to be spent on further evaluating the Anaspec kit and validation of the kit from Covance could be commenced. Validation of the commercial kit could be performed as planned and when the new antibody had been delivered experiments with the oligomeric assay could be continued.
Chapter 8

Conclusions and Proposal for future work

In this thesis, a commercial ELISA was validated and subsequently used to characterize a pre-clinical model of PD. Levels of α-syn was found to be significantly higher 4 weeks compared to 2 weeks post injection. This provides information for the future studies with the MPO inhibitor, which is believed to be neuroprotective. A novel ELISA assay was also developed and optimized. Measurements of oligomeric levels of α-syn in the pre-clinical model of PD could not be conducted due to sample limitations. Oligomeric forms of α-syn might prove to be valuable as a diagnostic biomarker, the value as a progression marker also need to be evaluated. In this thesis a method to measure these levels is provided.

The knowledge of PD and the contributing factors to the disease is increasing. To understand how levels of α-syn is related to the progression of PD, more research is needed. The lack of widely used sample processing and standard operating procedures is increasing the difficulty, making it hard to compare data from different groups. PD and other neurodegenerative diseases can probably never be explained by a single biomarker as the complexity and diversity of symptoms is high. A panel of biomarkers is probably needed where each can provide a share of the information needed to understand and follow the progression of PD and help in diagnosis and treatment. This approach have already been proved valuable in cancer research.

Earlier detection and hence, earlier disease intervention in combination with the emerging field with stem cell treatment, might provide a solution to the complexity of neurodegeneration in the future.
Bibliography


