Development and Validation of an in-vivo Bioassay

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Outline

- Background
- Feasibility studies
- Comparability study
- Prevalidation study
  - Design
  - Analysis
Background
Biological principle

- Factor Xa: important protein in blood coagulation system
- Many compounds for inhibition of Factor Xa are developed
- Medical need: reverse inhibition of Factor Xa for emergency operation

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Background

Biological principle

- Biotin (Vitamin H) / Avidin (Protein from egg): strongest known non-covalent bound

- Factor Xa inhibitors are biotinylated and can now interact with Avidin
Avidin used for in-vivo neutralization of biotinylated compounds

- Treatment of patients with antithrombotic compound
- Anti-Xa activity proportionally reversed by the applied dose of Avidin

Measured response: plasma level of active Anti-Xa compound over time
Background

Bioassay definition

Definition

„The experimental determination of the potency or strength of a chemical or biological substance based on the response observed after its administration to living matter (animal or derivatives) constitutes a biological assay.“

Objectives

„The primary purpose of bioassay is to estimate and compare the potencies of chemical or biological compounds under investigations based on appropriate well-designed experiment. The estimation and comparison of potencies must be accomplished by appropriate statistical methodologies.“

Quantitative bioassay

“A quantitative biological test is an experiment intended to quantify the activity of a substance through a biological reaction. As living creatures show inherent, fundamental variability, the biological activity of a substance cannot be characterized in absolute terms solely by its physicochemical parameters. The conditions under which the substance is used have been specified as precisely as possible, its activity remains variable from one moment to another.”
Background
Aim of bioassay method

- To determine the potency of Avidin reference standards

- To analyze and to compare the specific activity (to reverse Factor Xa-inhibition) of Avidin after modification of the production process (e.g. site change, scale up, ...)

- To demonstrate the stability of Avidin drug substance/drug product batches
Feasibility studies

- Definition of
  - Time range
  - Statistical end-point
  - Dose range
    - Linear log(dose)-response relationship

- Study 1:
  - Dose selection for antithrombotic compound

- Study 2, 3:
  - Dose selection Avidin
  - Statistical endpoint
  - Time range
  - Design for comparability study

- Study 4:
  - Proof of concept, stressed (deactivated) batches
Feasibility studies

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Feasibility study 1

- Rabbit bioassay model

- Dose response study for antithrombotic compound in rabbits
  - identify the optimal dose for Factor Xa inhibition
  - (0.02, 0.05, 0.1, 0.2, 0.5 mg/kg and negative control)

- Selected dose: 0.2 mg/kg (representative for clinical situation)
Feasibility study 1

Antithrombotic compound in µg/mL in rabbit plasma after IV administration (anti-Xa assay)

- Negative control
- Group 2 (0.02 mg/kg/D)
- Group 3 (0.05 mg/kg/D)
- Group 4 (0.1 mg/kg/D)
- Group 5 (0.2 mg/kg/D)
- Group 6 (0.5 mg/kg/D)

Plasma level (µg/mL) vs. time (minutes)

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Feasibility studies 2/3

- Definition of
  - Time range
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    - Linear log(dose)-response relationship

- Study 1:
  - Dose selection antithrombotic compound

- Study 2, 3:
  - Definition of statistical endpoint
  - Dose selection Avidin
  - Time range
  - Design for comparability study

- Study 4:
  - Proof of concept, stressed (deactivated) batches

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Feasibility studies 2

- 7 groups with 6 animals each

- Pretreatment at 0 min with antithrombotic compound
  - 0.2 mg/kg

- Avidin treatment at 60 min.
  - Negative control: 0 mg/kg
  - 0.3 – 30 mg/kg
  - Positive control: 0 mg/kg compound plus 30 mg/kg Avidin

- Compound plasma levels until 360 minutes

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Feasibility study 2

Compound in µg/mL in rabbit plasma after IV (0.2mg/kg) administration followed by Avidin administration at different dose levels (anti-Xa assay)

- positive control
- negative control
- group 3 (0.3 mg / kg)
- group 4 (1 mg / kg)
- group 5 (3 mg / kg)
- group 6 (10 mg / kg)
- group 7 (30mg / kg)

Plasma level (µg/mL) vs. time (minutes)

5' BT 15' 30' 55' 65' 75' 90' 120' 180' 240' 360'

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Feasibility study 2
Definition of end-point

- Area under the curve (AUC) for different time ranges
  - first value: 55 min (last sample before application of Avidin)
  - last value: 75, 90 or 120 minutes
  - normalization with negative control

- Ratio of plasma levels
  - Plasma level at 75, 90 or 120 minutes
  - Normalization with level at 55 minutes
Feasibility study 2

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Dose range:
- At ~3 mg/kg the lower asymptote is reached
- Clear non-linear dose-response relationship for all selected end-points, analysis with 4-parametric logistic model
- Linear log(dose)-response-relationship below 1-2 mg/kg

Statistical endpoint:
- AUC is more appropriate than just plasma level ratios, gives more accurate representation of the development between initial and last times
- Time range: 55 to 90 minutes (decrease in control group for later times; main effect from 55-65 min)
- Blood samples: 55, 65, 75, 90, 120 minutes
Feasibility study 3

- 7 groups with 6 rabbits each

- Pretreatment at 0 min with antithrombotic compound
  - 0.2 mg/kg

- Avidin treatment at 60 min.
  - Negative control: 0 mg/kg
  - 0.16 – 3.2 mg/kg
  - Positive control: 0 mg/kg compound plus 30 mg/kg Avidin

- Plasma levels until 120 minutes
Experimental Study on the Effect of Avidin on Coagulation Parameters (Factor X) in pretreated New Zealand White Rabbits

- Negative control
- Group 2 (0.16mg/kg)
- Group 3 (0.4mg/kg)
- Group 4 (0.8mg/kg)
- Group 5 (1.6mg/kg)
- Group 6 (3.2mg/kg)
Feasibility study 3
Selection of concentrations

- Clear non-linear dose-response relationship

- Analysis with 4-parametric logistic model
  - Upper asymptote: negative control group
  - Calculation of EC20, EC50, EC80
  - Select 3 Avidin concentrations for reversion of Anti-Xa inhibition of 20%, 50% and 80%

- Ensure linear log(dose)-response relationship for comparability study
Feasibility study 3
Selection of concentrations

- **Study 2: linear log(dose)-response-relationship below 1-2 mg/kg**
- **Fine-tuning with lower concentrations**
  - **EC20-EC50-EC80 values:**
    - 0.4, 0.6 and 1 mg/kg

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### Ratio of AUC (55-90 min)

<table>
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<th>Value</th>
<th>Description</th>
<th>Emin</th>
<th>EminF</th>
<th>Bottom</th>
<th>Weight</th>
<th>Iterations</th>
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<td>EC50 rel</td>
<td>1.60E-01</td>
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<td>3.58E-01</td>
<td>4.32E-01</td>
<td>4.6%</td>
<td></td>
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</tr>
</tbody>
</table>

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Equivalence of six test batches to a reference standard

Originally planned, separate runs per test batch

- Per run (repeated twice): 3 dose groups for test, 3 for standard batch, 1 negative control
- 6 test batches => 12 runs (overall 84 groups)
- Sample size calculations: rabbits per group

Performed, all batches together

- Per run: 1 negative control, 3 doses for 6 test batches and the standard batch (21 Avidin groups)
- 3 rabbits per group => 66 rabbits per run
- Sample size calculations: number of runs

6 per group => 504 rabbits
4 per group => 336 rabbits
4 runs => 264 rabbits
Feasibility studies 2/3

- Definition of
  - Time range
  - Statistical end-point
  - Dose range
    - Linear log(dose)-response relationship

- Study 1:
  - Dose selection compound

- Study 2, 3:
  - Definition of statistical endpoint
  - Dose selection Avidin
  - Time range
  - Design for comparability study

- Study 4:
  - Proof of concept, stressed (deactivated) batches
Feasibility study 4, Proof of concept

- 9 Avidin groups and 1 negative control
  - Groups 2-4: dose groups Avidin standard
  - Groups 5-7: dose groups Avidin stressed by temperature and acid pH
  - Groups 8 to 10: dose groups Avidin stressed by 50% biotin neutralization

- Method can identify stressed (poor) batches

- Doses for future studies adapted to: 0.5, 0.8 and 1.1 mg/kg
Feasibility study 4, Proof of concept

Experimental study on the effect of stressed Avidin samples on anti-Xa activity in pretreated New Zealand White rabbits

Plasma level (µg/ml) vs. time (minutes)

- Negative control
- Gr.2 Avidin QC3 (0.4mg/kg)
- Gr.3 Avidin QC3 (0.7mg/kg)
- Gr.4 Avidin QC3 (1.1mg/kg)
- Gr.5 Avidin QC2/1 (0.4mg/kg)
- Gr.6 Avidin QC2/1 (0.7mg/kg)
- Gr.7 Avidin QC2/1 (1.1mg/kg)
- Gr.8 Avidin QC1/2 (0.4mg/kg)
- Gr.9 Avidin QC1/2 (0.7mg/kg)
- Gr.10 Avidin QC1/2 (1.1mg/kg)
Prevalidation study, Objective

- Validation of standard (vs. itself)
- Variance model for weighted fit
- Equivalence limits for parallelism
Prevalidation study, Design

- 5 sets of 3 assays each

- 1 assay includes
  - 3 “reference” groups: 0.5, 0.8 and 1.1 mg/kg
  - 3 “test” groups: 0.5, 0.8 and 1.1 mg/kg
  - 1 negative control

- 3 rabbits per group, both reference and test will be working standard
Prevalidation study

Dose response curve fit

- Linear regression: \( y = a + b \times x \)

Variance model for weighted fit:

- \( \text{VAR}_{ij} = A \times \text{MEAN}_{ij}^B \)
- \( \ln(\text{VAR}_{ij}) = \ln(A) + B \times \ln(\text{MEAN}_{ij}) \)

Parameters estimated from 90 values:
- (5 runs * 3 assays * 2 compounds * 3 doses)

Weights: \( w_i = 1 / \text{Var}(Y_i) = 1 / (A \times Y_i^B) = 1 / (0.0025 \times Y_i^{0.9121}) \)

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Parallelism metric:

- **Free model**: independent fit for standard and test batches
  - \( y_{STD} = a_{STD} + b_{STD} \cdot \log(x_{STD}) \)
  - \( y_{UK} = a_{UK} + b_{UK} \cdot \log(x_{UK}) \)

- **Constrained model**: the same slope and intercept
  - \( y_{STD} = a_{C} + b_{C} \cdot \log(x_{STD}) \)
  - \( y_{UK} = a_{C} + b_{C} \cdot (\log(x_{UK}) + \log(r)) \)

- **Shift r (relative potency)**: \( r = x_{STD}/x_{UK} \)

- **Parallelism metric**: difference between squared residuals of constrained and free model
  - Calculation of upper limit ULK from prevalidation data base
  - \( ULK = \chi^2(1-\alpha,p) = 4.9 \)
Routine testing for batch release

- **Design for routine testing**
  - 3 rabbits per group, 3 dose groups each for standard and test, 1 negative control

- **Suitability**
  - Comparison of parallelism statistics to $\text{ULK}=4.9$

- **Relative potency**
  - Calculation of $r$ with 95%CI using constrained model

- **Trueness and precision**
  - Precision of the method can be compared to prevalidation study results
Conclusion

- Very consistent step-by-step experimental and statistical approach
  - 4 parametric logistic fit, selection of doses, time range and statistical endpoint
  - Sample size calculations and design approach (for comparability) to allow:
    - Reduction of number of rabbits
    - Post-hoc additional runs in case of low power without additional (unconsiderable) variability
    - Statistical sound comparison of batches with or without parallelism approach
  - Equivalence instead of difference approach
- Procedure can be used as general strategy for development of future bioassays
Acknowledgement

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Thank you!
Feasibility studies 2/3
Design of Comparability study

- Preliminary Information:
  - Plasma concentrations at 55 minutes for all groups
  - 6 groups, 6 values each
  - Calculation of repeatability and intermediate precision CV:

- Results from study 2
  - Repeatability: CV=6.7%
  - Intermediate Precision: CV=7.3%
  - Ratio of CVs: 0.92

- Results for study 3
  - Repeatability: CV=5.0%
  - Intermediate Precision: CV=6.1%
  - Ratio of CVs: 0.82
Sample size calculations: 4 runs

- 22 groups per run (3 animals per group):
  - 3 reference groups, Avidin standard (reference doses as selected)
  - 3 groups per batch for all 6 batches
  - 1 additional negative control group

Measurements

- Statistical end-point: AUC from 55 to 90 minutes
- Blood samples: 55, 65, 75, 90 and 120 minutes
Comparability study, analysis

Comparability study on the effect of Avidin samples on anti-Xa activity in pretreated New Zealand White rabbits - a comparison between different Avidin batches

![Graph showing the effect of Avidin samples on anti-Xa activity in pretreated New Zealand White rabbits.](image)

- **Negative control**
- **Group 2 Avidin QC3 (0.5 mg/kg)**
- **Group 3 Avidin QC3 (0.8 mg/kg)**
- **Group 4 Avidin QC3 (1.1 mg/kg)**
- **Group 5 Avidin 1002-07 (0.5 mg/kg)**
- **Group 6 Avidin 1002-07 (0.8 mg/kg)**
- **Group 7 Avidin 1002-07 (1.1 mg/kg)**
- **Group 8 Avidin 1003-07 (0.5 mg/kg)**
- **Group 9 Avidin 1003-07 (0.8 mg/kg)**
- **Group 10 Avidin 1003-07 (1.1 mg/kg)**
- **Group 11 Avidin 1004-07 (0.5 mg/kg)**
- **Group 12 Avidin 1004-07 (0.8 mg/kg)**
- **Group 13 Avidin 1004-07 (1.1 mg/kg)**
- **Group 14 Avidin 7R00004 (0.5 mg/kg)**
- **Group 15 Avidin 7R00004 (0.8 mg/kg)**
- **Group 16 Avidin 7R00004 (1.1 mg/kg)**
- **Group 17 Avidin 7R00005 (0.5 mg/kg)**
- **Group 18 Avidin 7R00005 (0.8 mg/kg)**
- **Group 19 Avidin 7R00005 (1.1 mg/kg)**
- **Group 20 Avidin AR00001 (0.5 mg/kg)**
- **Group 21 Avidin AR00001 (0.8 mg/kg)**
- **Group 22 Avidin AR00001 (1.1 mg/kg)**

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Comparability study, Analysis

- Analysis per dose level
  - Reproducibility study
  - Calculation of variances within group, between runs and between batch
  - Acceptance criterion of 20%

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For all tested batches (3 batches from Frankfurt and 3 batches from Aramon), at the 3 Avidin doses, the bias with 90% confidence interval is below the acceptance criterion of 20%.

As a consequence, the relative difference of activity between the six batch samples and the standard sample being significantly lower than 20%, the activity of these batches may be declared as equivalent to the activity of the standard sample.