Development and Validation of an in-vivo Bioassay

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sanofi aventis
Because health matters

Outline

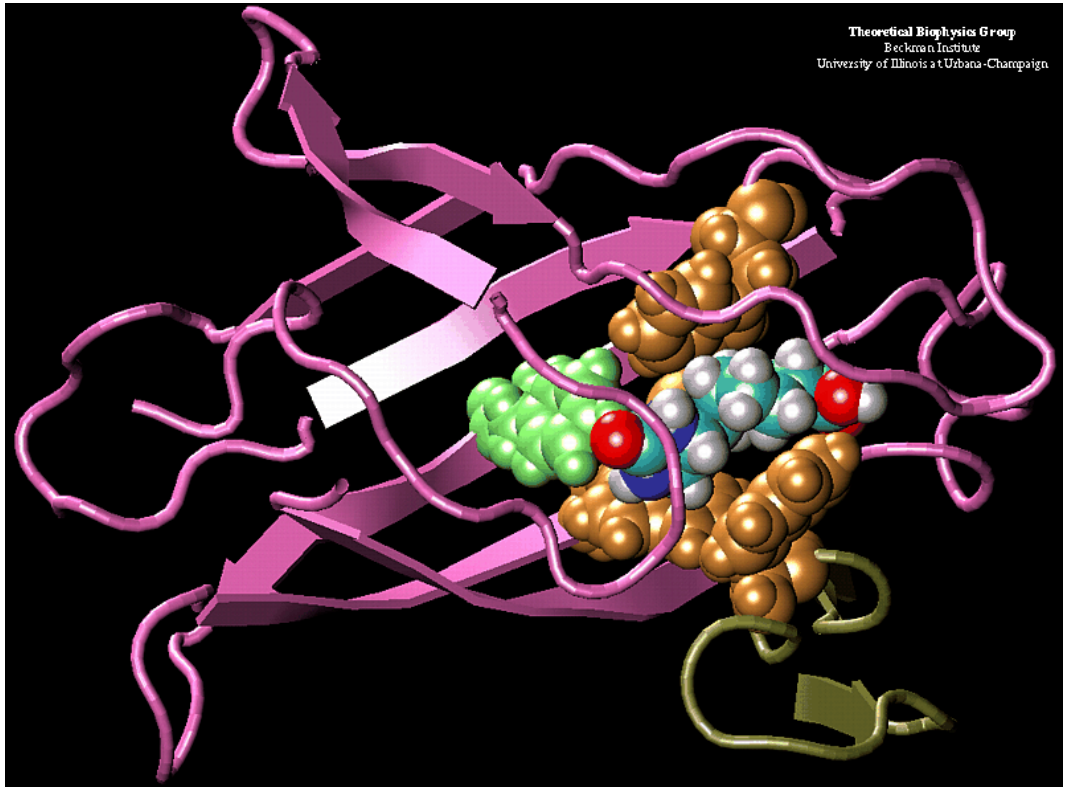
- Background
- Feasibility studies
- Comparability study
- Prevalidation study
  - Design
  - Analysis

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

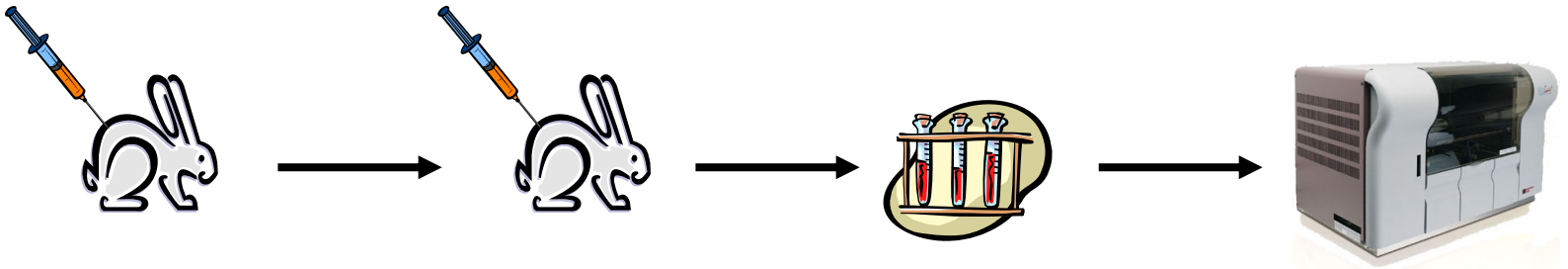
Background
Biological principle

- Biotin (Vitamin H) / Avidin (Protein from egg): strongest known non-covalent bound
- Factor Xa inhibitors are biotinylated and can now interact with Avidin



Background
Experimental process

- 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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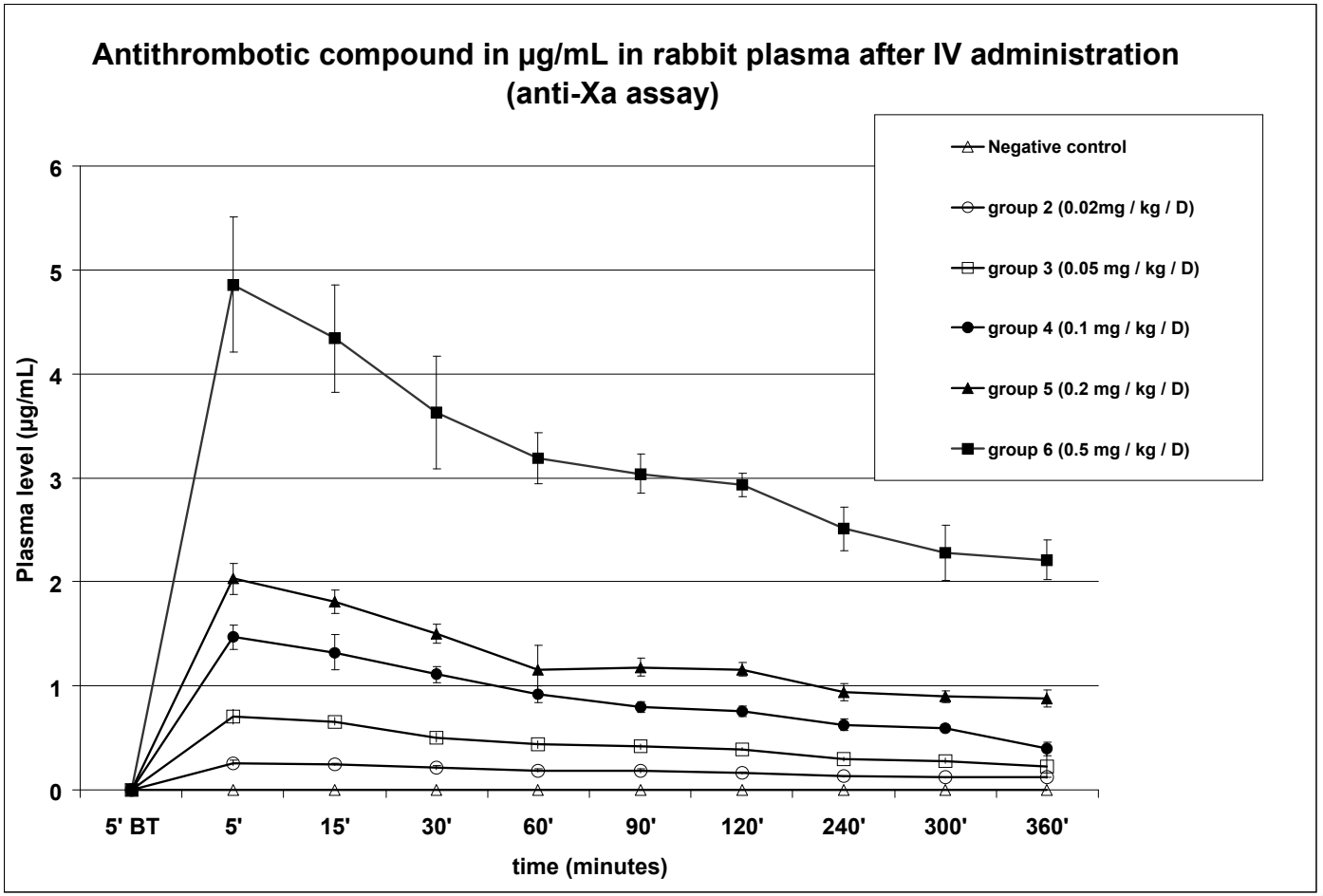
Feasibility study 1

- Rabbit bioassay model
- Dose response study for antithrombitic 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



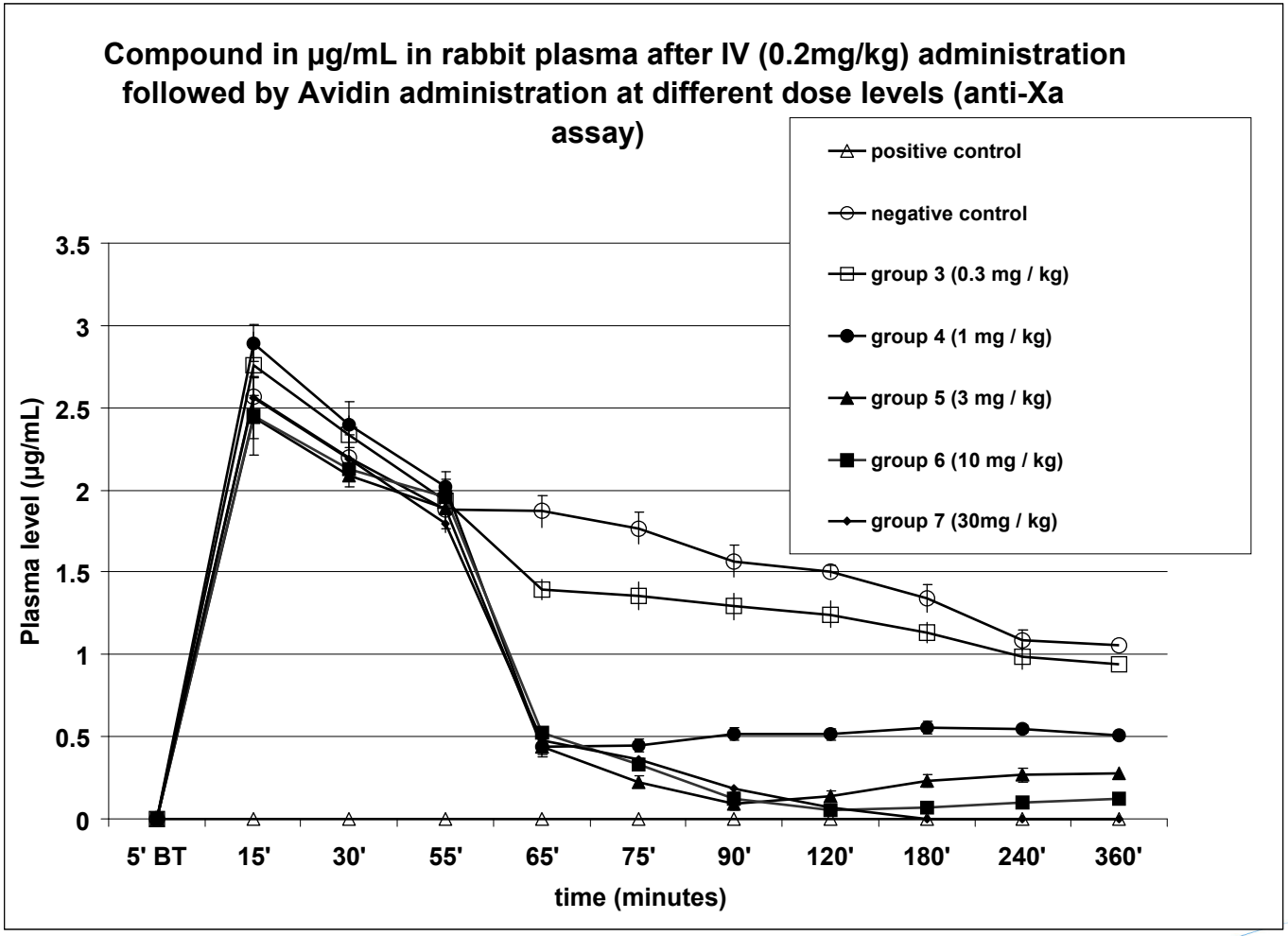
Feasibility studies 2/3

- Definition of
  - Time range
  - Statistical end-point
  - Dose range
    - 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

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

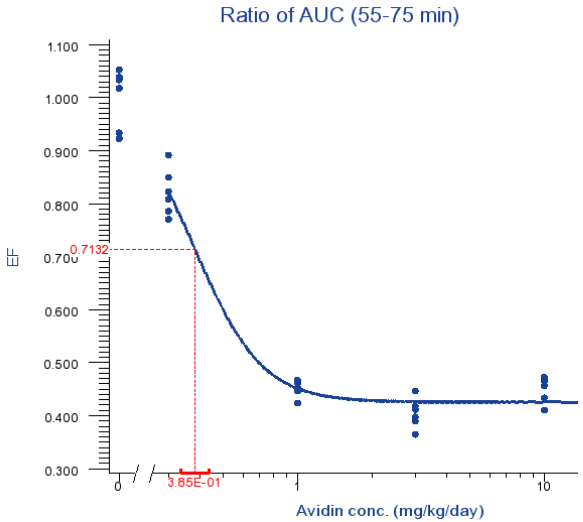
Feasibility study 2

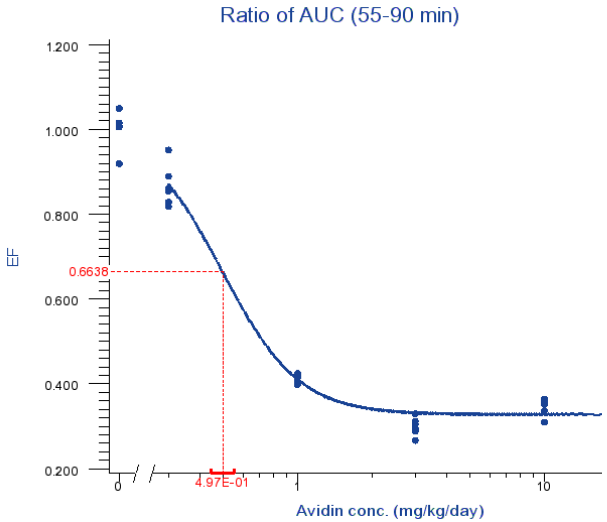


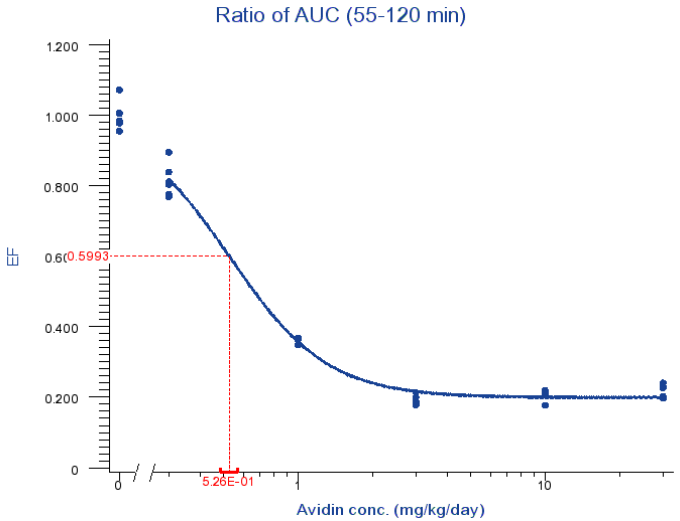
Feasibility study 2
Definition of end-point

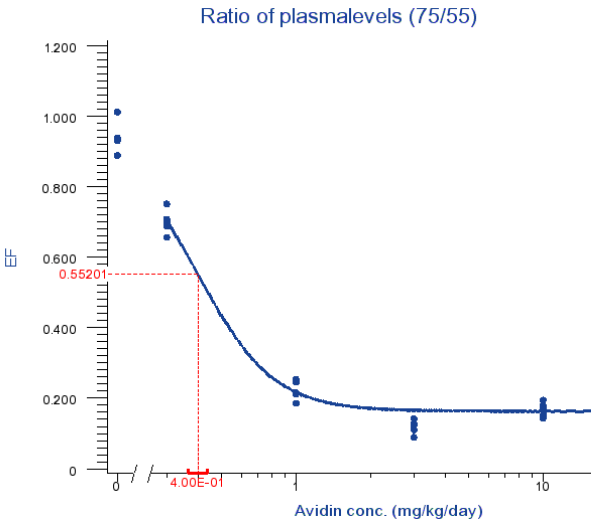
- Area under the curve (AUC) for different time ranges
  - first value: 55 min (last sample before application of Avidin)
  - last value: 75, 90 or 120 minutes
  - normalization with negative control
- Ratio of plasma levels
  - Plasma level at 75, 90 or 120 minutes
  - Normalization with level at 55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

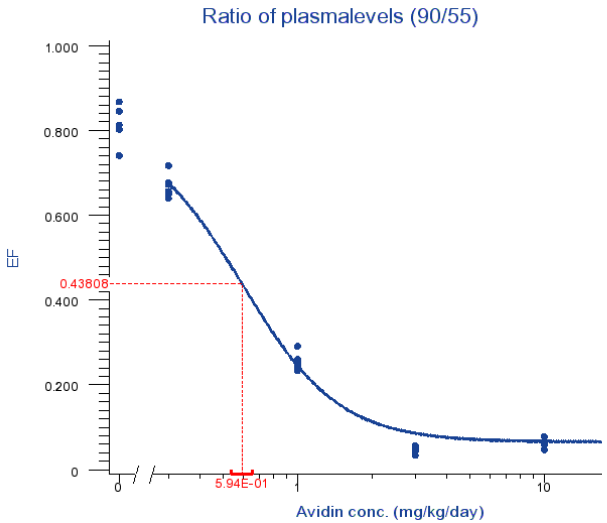
Feasibility study 2

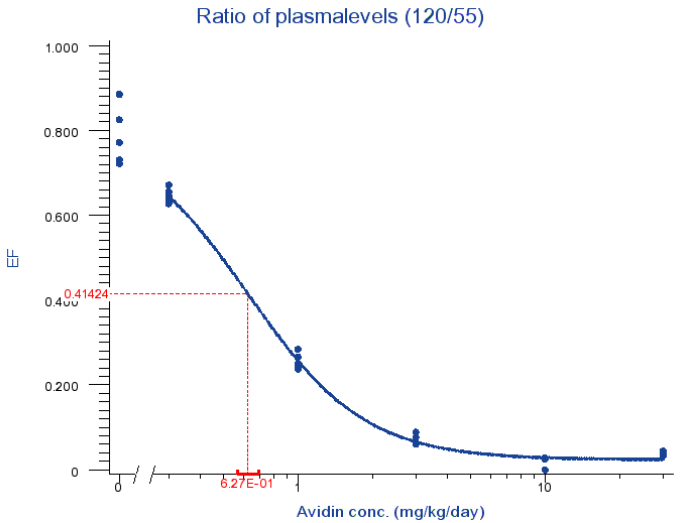












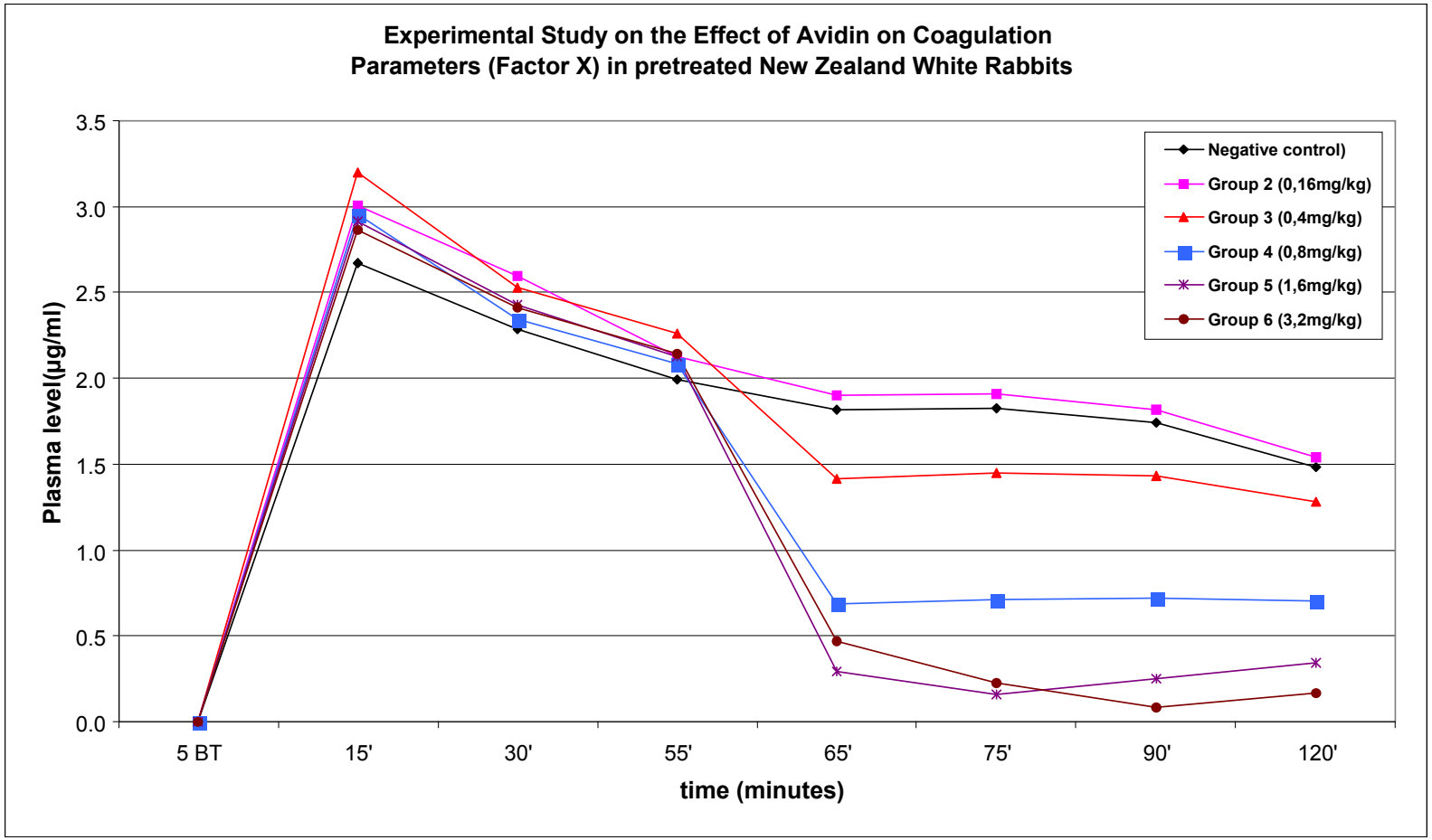
Feasibility study 2
Definition of end-point

- **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

Feasibility study 3

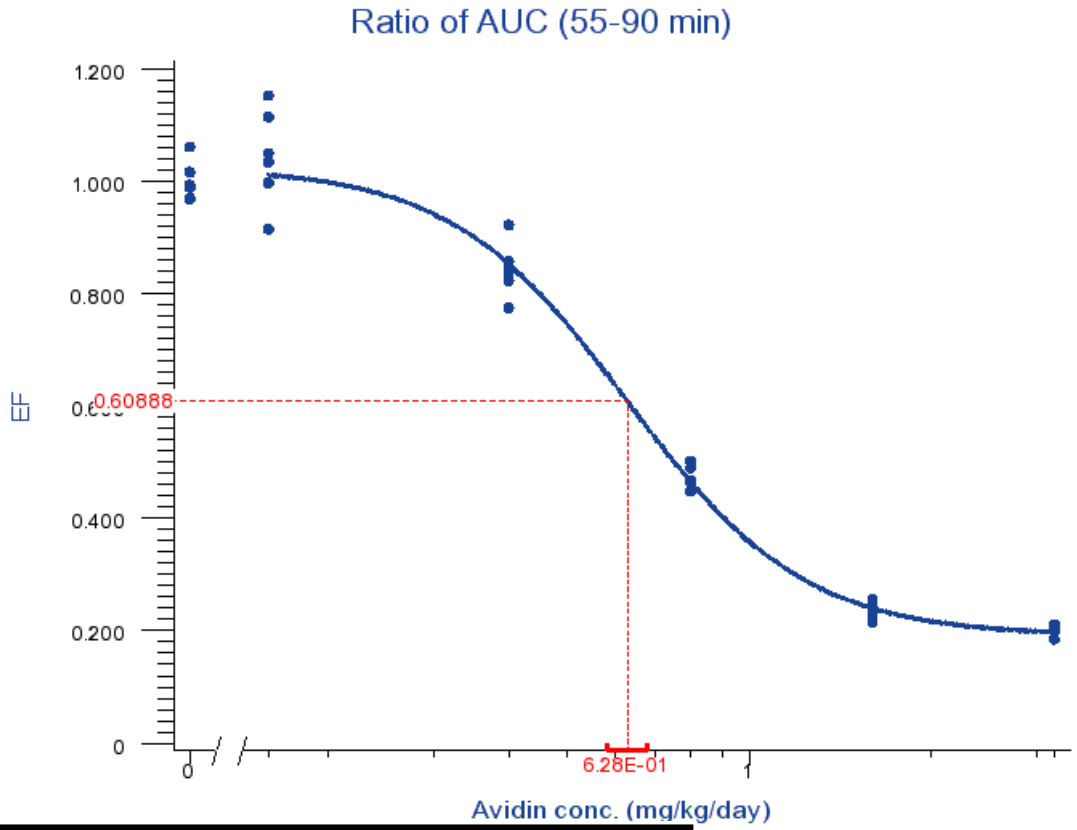


Feasibility study 3
Selection of concentrations

- 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



Ratio of AUC (55-90 min)									
EC50 rel	6.28E-01 [5.82E-01 ; 6.77E-01] CV= 3.7%	EminC	1.60E-01	EminF	1.01177	Bottom	0.19175	Weight	-
Y.50 rel	0.60888	EmaxC	3.20E+00	EmaxF	0.19837	Top	1.02601	Iterations	7
EC20 rel	1.00E+00 [9.19E-01 ; 1.09E+00] CV= 4.2%					Slope	-2.97	SSE	0.068446
EC80 rel	3.93E-01 [3.58E-01 ; 4.32E-01] CV= 4.6%								

Feasibility studies 2/3
Design of Comparability study

- 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

6 per group
=>504 rabbits

4 per group
=>336 rabbits

- 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

4 runs

=>264 rabbits

Feasibility studies 2/3

- Definition of
  - Time range
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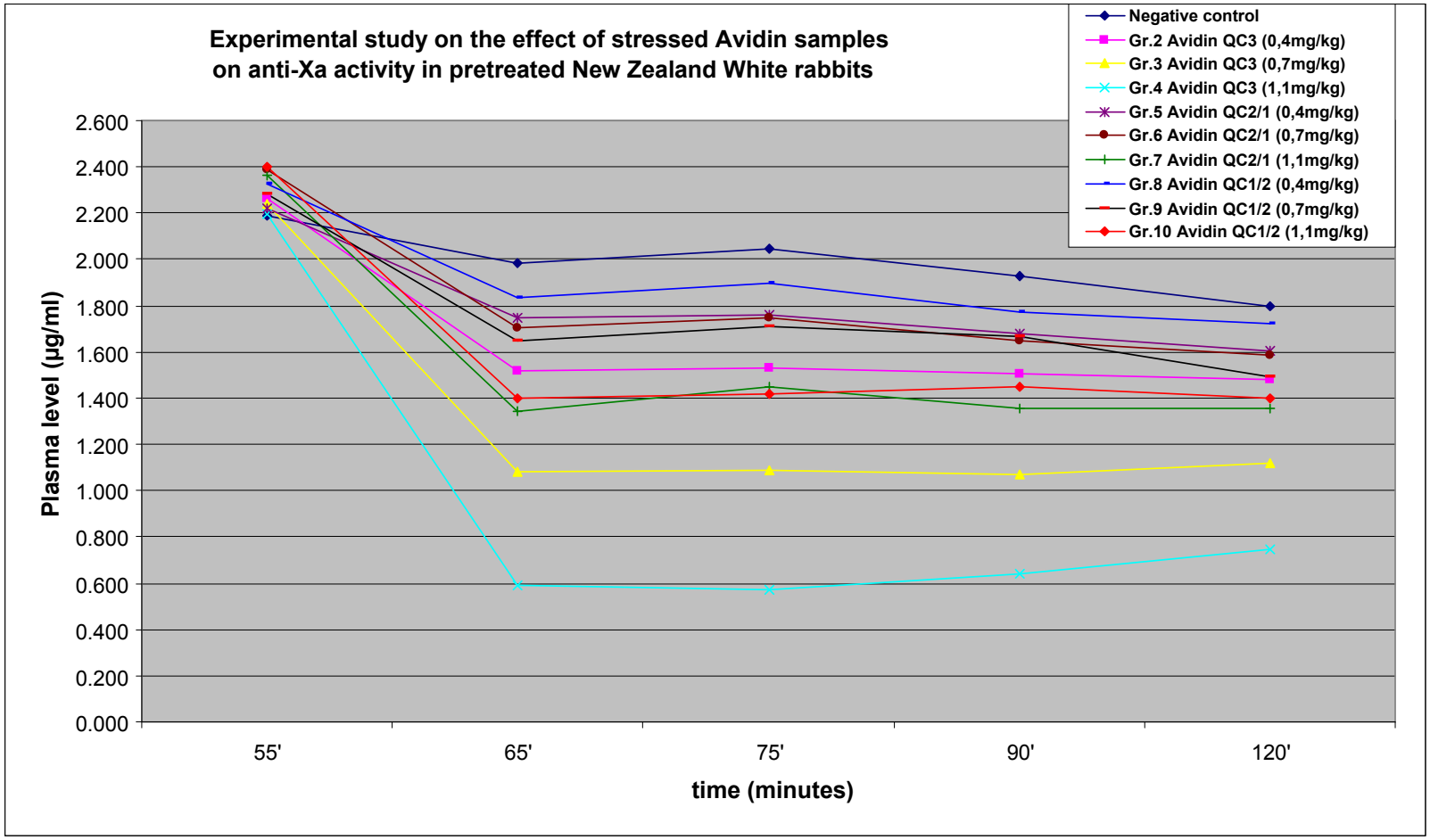
  - Dose range
    - Linear log(dose)-response relationship
- Study 1:
  - Dose selection compound
- Study 2, 3:
  - Definition of statistical endpoint
  - Dose selection Avidin
  - Time range
  - Design for comparability study
- Study 4:
  - Proof of concept, stressed (deactivated) batches

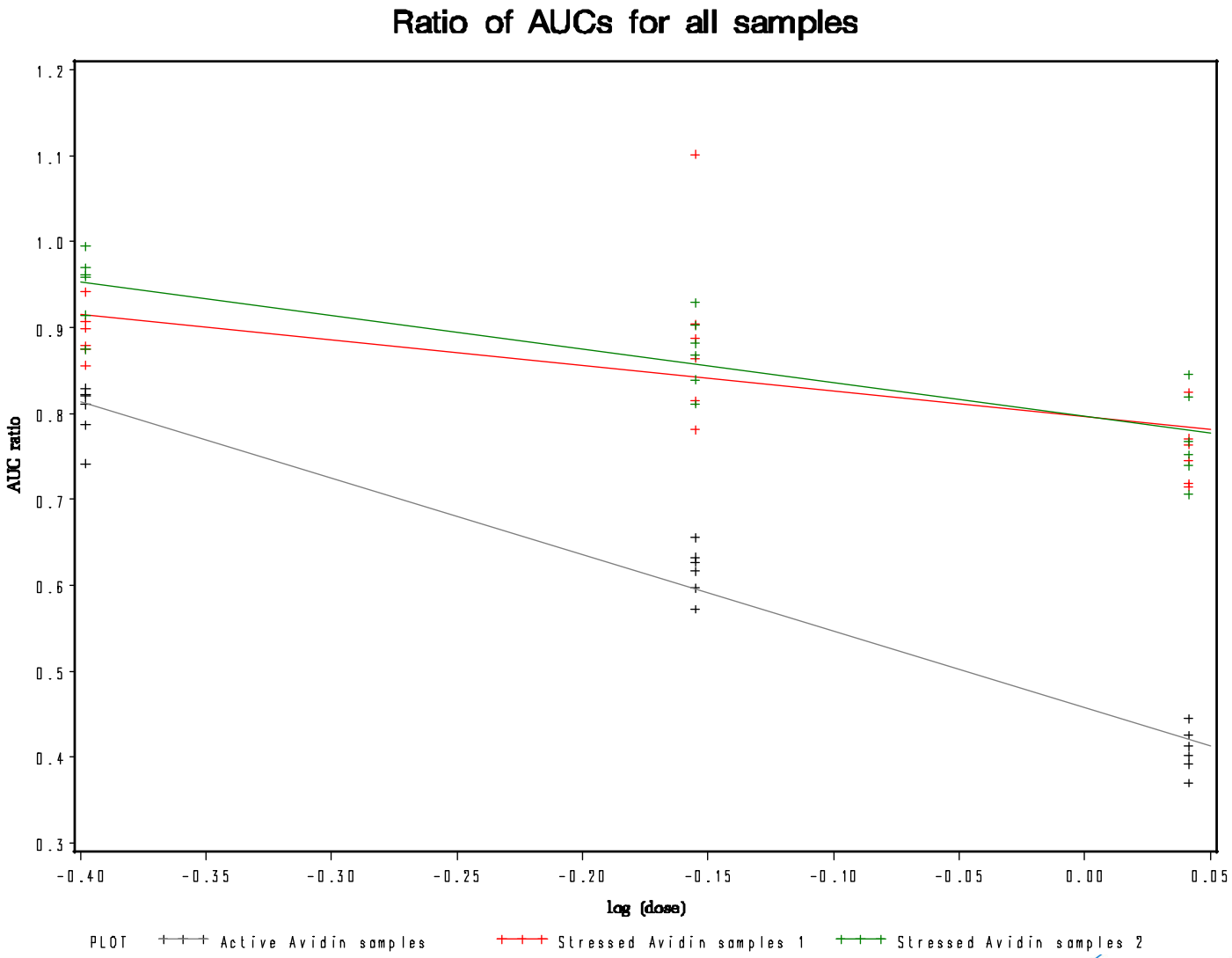
Feasibility study 4, Proof of concept

- 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



Feasibility study 4, Proof of concept



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

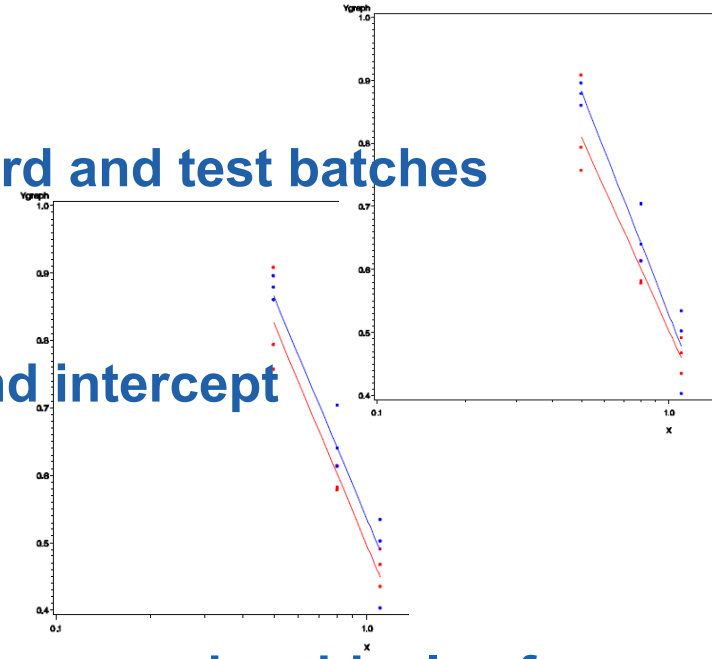
Dose response curve fit

- Linear regression: y=a+b*x
- Variance model for weighted fit:
  - $VAR_{ij}=A*MEAN_{ij}^{B}$
  - $\ln(VAR_{ij})=\ln(A)+B*\ln(MEAN_{ij})$
  - Parameters estimated from 90 values:
    (5 runs * 3 assays * 2 compounds * 3 doses)
  - Weights: $w_i=1/Var(Y_i)=1/(A*Y_i^{B})=1/(0.0025*Y_i^{0.9121})$

Prevalidation study

Parallelism metric:

- **Free model: independent fit for standard and test batches**
  - $y_{STD}=a_{STD}+b_{STD}*\log(x_{STD})$
  - $y_{UK}=a_{UK}+b_{UK}*\log(x_{UK})$
- **Constrained model: the same slope and intercept**
  - $y_{STD}=a_C+b_C*\log(x_{STD})$
  - $y_{UK}=a_C+b_C*(\log(x_{UK})+\log(r))$
- **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 **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

Christophe Agut

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Thank you!

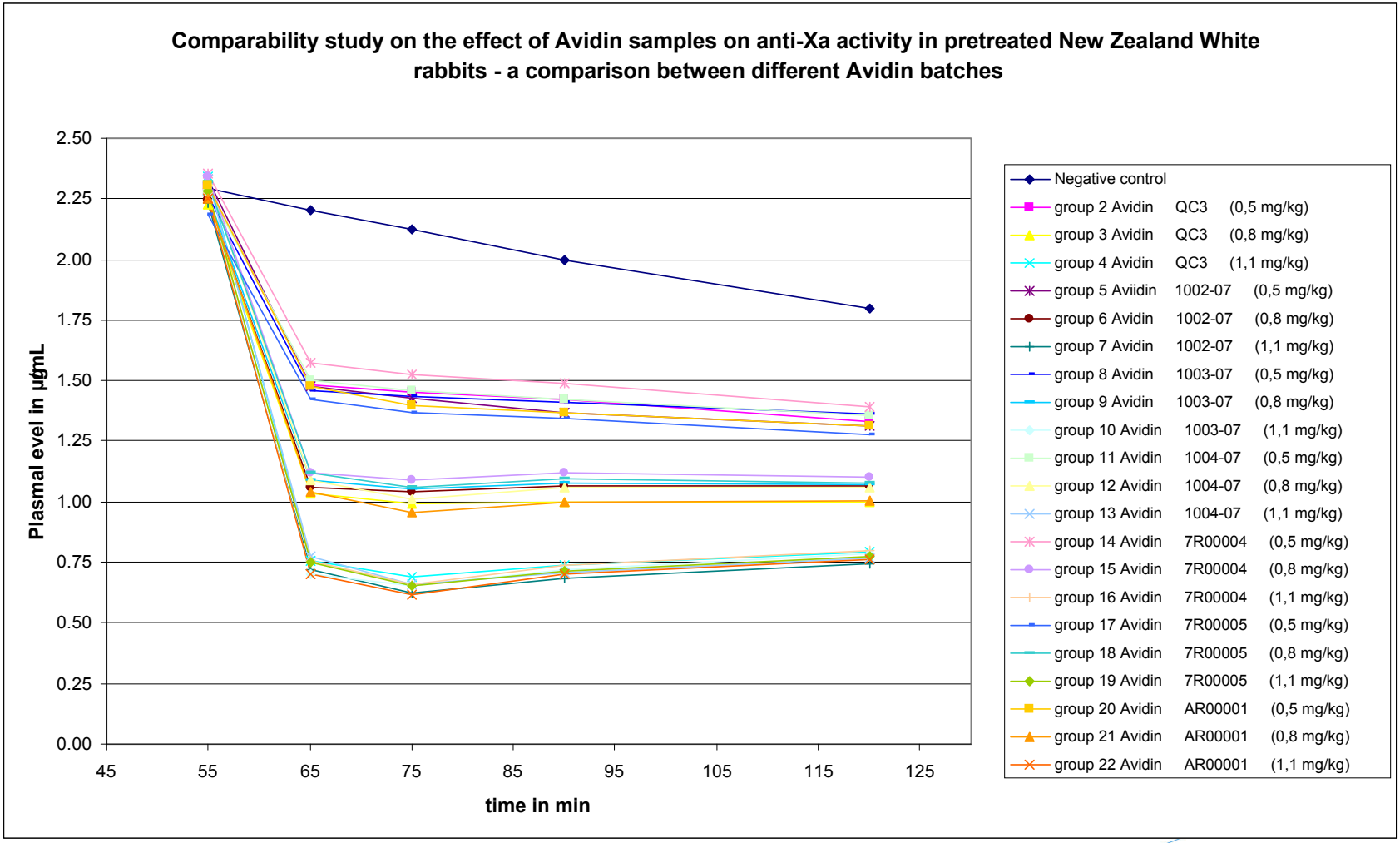
Feasibility studies 2/3
Design of Comparability study

- **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**

Feasibility studies 2/3
Design of Comparability study

- 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, Analysis

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

Comparability study, Results

- 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.