Analytical Development and Validation

Helmut Schütz
BEBAC

Main Topics

- Validation = Suitability for Use?
  - Method development – which Analyte?
  - Matrix Effects in LC/MS-MS
  - Ligand Binding Assays
- Method Validation (Arlington Conferences I-III)
  - Validation Plan
  - Pre-Study Validation
  - Validation Report
  - Analytical Protocol / In-Study Validation
- Plausibility Review
- Open Issues

Chiral *vs.* achiral

- EMA GL on BE (2010, Section 4.1.5)
  - Achiral methods generally acceptable
  - Chiral methods, if *all* conditions are met *or are unknown*:
    1. Enantiomers exhibit different pharmacokinetics.
    2. Enantiomers exhibit pronounced differences in pharmacodynamics.
    3. The exposure (AUC) ratio of enantiomers is modified by a difference in the rate of absorption.
  - If only one enantiomer is active and the other is inactive or has low contribution to activity, it is sufficient to demonstrate BE for the active only.

Parent *vs.* Metabolite

- EMA GL on BE (2010, Section 4.1.5)
  - Generally parent drug.
  - Concerns, that C_{max} of metabolite does not reflect difference between formulations.
  - Does not distinguish between active and inactive metabolites.
  - If method too insensitive consider higher SD.
  - Metabolite acceptable *'in exceptional cases'*:
    - Present any available data supporting the view that the metabolite exposure will reflect parent drug.
    - Metabolite formation is not saturated at therapeutic doses.

Pro-drugs

- EMA GL on BE (2010, Section 4.1.5)
  - Generally inactive pro-drug.
  - Active does not need to be measured.
  - If pro-drug has low concentrations and is quickly eliminated it is acceptable to demonstrate BE of active metabolite (pro-drug not measured).
  - Parent compound can be considered to be an inactive pro-drug if it has no or very low contribution to clinical efficacy.

Assumptions: Bioanalytics

- Assumptions should – possibly – be justified in method development, *e.g.*,
  - Absence of co-eluting compounds (MS-MS < MS < FL < EC < UV-DAD < UV)
  - Lack of Matrix Effects (LC/MS-MS, Ligand Binding Assays)

$$MF = \frac{\text{peak response in presence of matrix ions}}{\text{peak response in mobile phase}}$$

Matrix Factor ~1

  - Protein-binding (generally only total concentration [free+bound] measured)

Validating Methods

- Methods used for quantitative measurement of analytes in any given biological matrix must be

reliable and reproducible ***for the intended use…***

- Accuracy
- Precision
- Selectivity
- Sensitivity
- Reproducibility
- Stability

- C_{max} (ULOQ)
- $AUC_t/AUC_\infty \geq 80\%$ (LLOQ)

BE 5% of C_{max}!

- 15–20% Bias / Precision (BE ↔ sparse sampling Population PK)

Validating Methods

- Level of Regulations
  - Non-clinical studies: GLP
  - Clinical studies:
    - FDA: non-GLP
    - EU: NfG on BA/BE (2002): The bioanalytical part of bioequivalence trials should be conducted accord-ing to the applicable principles of Good Laboratory Practice (GLP).

      GL on BE (2010): […] However, as human bioanalytical studies fall outside the scope of GLP, the sites conducting the studies are not required to be monitored as part of a national GLP compliance programme.

Validating Methods

- Reference standard
  - FDA
    - If possible, identical to the analyte.
    - If not, an established chemical form (free base or acid, salt or ester) of known purity can be used.
    - Types
      - Certified reference standards (*e.g.*, USP compendial standards)
      - Commercially supplied reference standards obtained from a reputable commercial source
      - Other materials of documented purity custom-synthesized by an analytical laboratory or other non-commercial establishment.

Validating Methods

- Reference standard
  - EU (applying OECD-GLPs)
    - Each […] item should be appropriately identified (*e.g.*, code, Chemical Abstracts Service Registry Number [CAS number], name, …).
    - For each study, the identity, including batch number, purity, composition, concentrations, or other characteristics to appropriately define each batch […] should be known.
    - In cases where the test item is supplied by the sponsor, there should be a mechanism, developed in co-operation between the sponsor and the test facility, to verify the identity of the test item subject to the study.
    - The stability of […] items under storage […] conditions should be known for all studies.

Validating Methods

- Reference standard
  - EU (Draft 2009)
    - Obtained from an authentic and traceable source.
    - Suitable reference standards, include certified standards such as compendial standards (EPCRS, USP, WHO), commercial available standards, or fully characterised standards prepared in-house or by an external non-commercial organisation.
    - Suitability of the reference standard should be scientifically justified.

Validating Methods

- Reference standard
  - FDA
    - The source and lot number, expiration date, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard.

Validating Methods

- Reference standard
  - EU (Draft 2009)
    - The use of certified standards is not needed for IS, as long as the suitability for use is demonstrated, e.g. lack of interference is shown for the substance itself or any impurities thereof.
    - Whoever the supplier, a certificate of analysis is required to ensure quality, stability, storage conditions, expiration date, batch number and purity of the reference standards.

Validating Methods

- Approaches to examine Matrix Effects
  - Extract various lots of blank matrix, add a constant amount of analyte and internal standard and plot the ratio for each lot. If the ratio remains constant, the matrix effect is insignificant.
  - Compare the slopes of calibration curves prepared in different sources of matrix.
  - Infuse low levels of analyte post column while injecting reconstituted extracted matrix on the LC. This allows the observation of matrix effects under various chromatographic conditions.

Validating Methods

- Minimization of Matrix Effects
  - Use IS of similar structure (preferably stable isotope – labeled; recommended by EMA 2009).
  - Avoid *'precipitate and shot'*–methods.
  - Conduct sufficient sample cleanup – especially to remove phospholipids.
  - Use new chromatographic methods (Ultra Performance LC, Rapid Resolution LC) to enhance separation.
  - Use weak acid wash solution for on-line SPE negative ion methods to break up Na^+/analyte ion pairs.
  - Maintain a clean MS source!
  - Consider APCI or FAIMS.
  - If everything fails, consider GC/MS!

Validation Plan

- Written Document describing which steps will be performed in the Validation.
  - Purpose of Validation (*e.g.*, 'Validation of bio-analytical method ***X*** for the determination of ***Y*** in matrix ***Z***').
  - Reference to established method (working instruction, SOP).
  - If another document exists, already describing the usal steps in validation – cross-reference is enough – otherwise detailed descriptions are necessary.

Pre-Study Validation

- **Terminology**
  - **Specificity *vs.* Selectivity** (IUPAC)
    - Specific is considered to be the ultimate of *selective*, meaning that no *interferences* are supposed to occur.
    - selective (in analysis)
      A term which expresses qualitatively the extent to which other substances interfere with the determination of a substance according to a given procedure.
  - *Specificity* is a rather theoretical state; in the real world we should assess *selectivity* only – which depends on the analyte, metabolites, degradents, co-administered compounds, matrix components,…

S Bansal and A DeStefano
Key Elements of Bioanalytical Method Validation for Small Molecules
The AAPS Journal 9(1), E109-E114 (2007)
http://www.aapsj.org/articles/aapsj0901/aapsj0901011/aapsj0901011.pdf

Pre-Study Validation

- **Full Validation**
  - **Selectivity** (FDA: mixed up with specificity)
    Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.
    - ≥6 sources of blank samples of the appropriate biological matrix (ANVISA: +1 hemolytic, +1 lipemic) should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).
    - Potential interfering substances: endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics.
    - Acceptable limit: ≤20% of response at LLOQ.

Pre-Study Validation

- **Full Validation**
  - **Selectivity** (cont'd)

    Matrix Effects in MS-based Assays
    - Matrix Factor

$$MF = \frac{\text{peak response in presence of matrix ions}}{\text{peak response in mobile phase}}$$

MF=1: no matrix effects
MF<1: ion suppression
MF>1: ion enhancement *or* analyte loss in the presence of matrix during analysis.

Pre-Study Validation

- **Full Validation**
  - **Selectivity** (cont'd)

    Matrix Effects in MS-based Assays
    - Suitability of internal standards (IS) in MS
      - Stable isotope – labeled IS:

        ^{2}H, ^{15}N, $^{18}0$ at 3-6 positions – different *m/z*, but similar extraction and chromatography.

        Should be used whenever possible!
      - Structural analog IS
        - Neutral radical (*e.g.*, $-CH_3$, $-C_2H_5$) preferred
        - Radicals of different polarity/pK less suitable (*e.g.*, -OH, NH_2), because extraction and/or chromato-graphy will be influenced.
      - Last resort: any other compound of similar polarity…

Pre-Study Validation

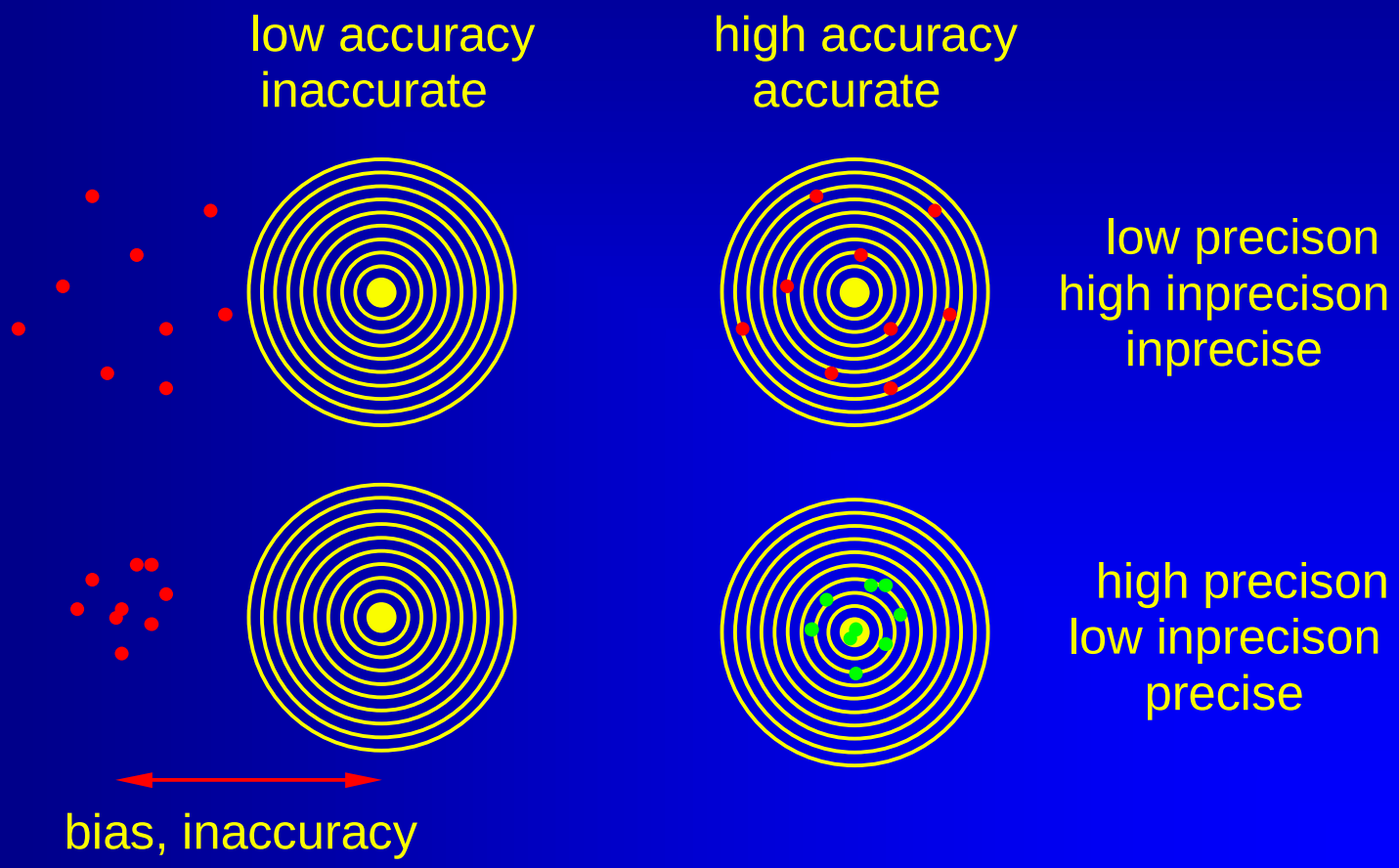
- **Full Validation**
  - **Selectivity** (cont'd)

    A MF of ~1 not necessary for a reliable bioanalytical assay. However, a highly variable MF in individual subjects would be a cause for the lack of reproducibility of analysis.
    - If no stable isotope – labeled IS is used,
      - to predict the variability of matrix effects in samples from individual subjects, MF should be determined in six individual lots of matrix.
        - Variability in matrix factors (measured by CV) should be less than 15%.
      - If the matrix is rare and hard to obtain, the requirement for assessing variability of MFs in six lots can be waived.

Pre-Study Validation

- **Full Validation**
  - **Selectivity** (cont'd)
    Do not forget separation of analyte and metabolite(s) in LC/MS-MS!
    - If using poor extraction and/or short run times:
      - in-source dissociation of:
        - acyl-glucuronides,
        - esters,
        - N-oxides,
        - lactone-rings.
      - In such a case interference is not the metabolite itself, but the resulting parent-compound itself!
      - Should be evaluated according to EMA Draft GL.

Pre-Study Validation



Pre-Study Validation

- **Full Validation** (cont'd)
  - **Precision**

    Replicate (≥5) analysis of known concentrations measured at ≥4 levels (LLOQ, low=≤3×LLOQ, intermediate, high).
    - Imprecision (CV%):

      ≤15% at each concentration (except at LLOQ, where ≤20% is acceptable.
    - Inaccuracy (absolute mean bias – RE%):

      ≤15% at each concentration (except at LLOQ, where ≤20% is acceptable.
    - Both parameters
      - intra-batch (within analytical run).
      - inter-batch (between analytical runs; aka repeatability).

Pre-Study Validation

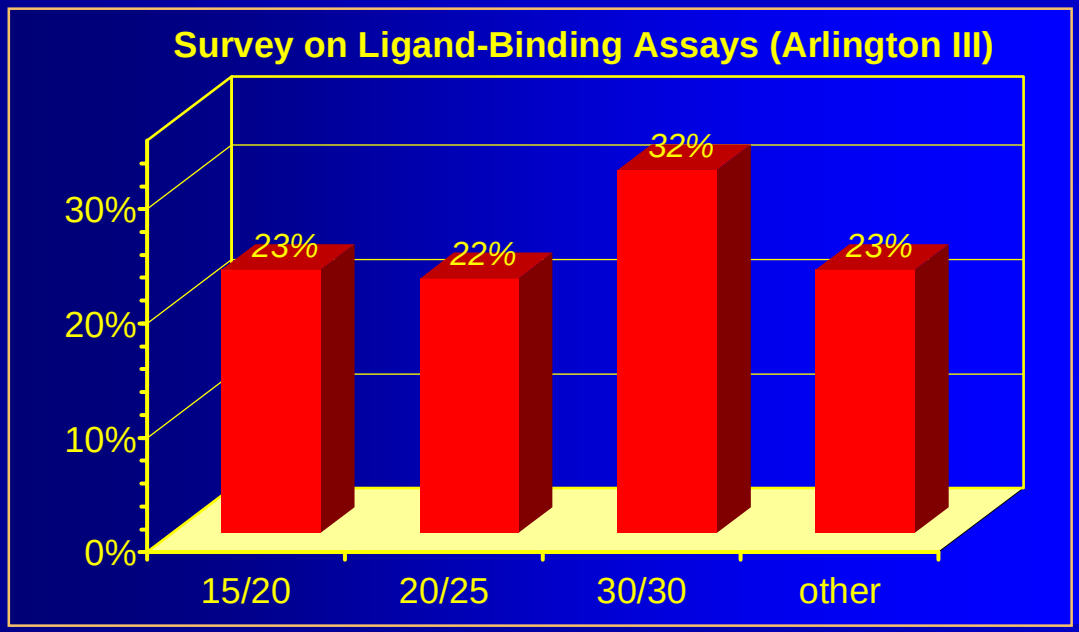
- **Full Validation** (cont'd)
  - **Precision**

    EMA Draft: 'To enable evaluation of any trends over time within one run, it is recommended to demonstrate accuracy of QC samples over at least one of the runs with a size equivalent to a prospective analytical run.'

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Precision** (cont'd)

    In 2006 problems were evident if trying to work according to FDA's bioanalytical guideline (2001)…



Pre-Study Validation

- **Full Validation** (cont'd)
  - **Precision** (cont'd)
    Ligand-binding assays according to Arlington III White-Paper:
    - Replicate (≥6) analysis of known concentrations measured at ≥5 levels in duplicate.
      - Anticipated LLOQ
      - ~3× LLOQ
      - Midrange (geometric mean of LLOQ and ULOQ)
      - High (~75% of ULOQ)
      - Anticipated ULOQ

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Precision** (cont'd)

    Ligand-binding assays according to Arlington III WP:
    - Inter-batch impression (CV%) and inaccuracy (absolute mean bias (RE%):
      - ≤20% at each concentration (except at LLOQ and ULOQ, where ≤25% is acceptable).
      - Target total error (sum of the absolute value of the RE% [accuracy] and inprecision [%CV%] should be less than ≤±30% [≤±40% at the LLOQ and ULOQ]). The additional constraint of total error allows for consistency between the criteria for pre-study method validation and in-study batch acceptance.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Recovery**
    - The detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard.
    - Recovery of the analyte does not need to be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible.
    - Measured at low/intermediate/high level.
    - Not required according to EMA Draft GL!

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Carry-over**
    - Only according to EMA Draft GL.
    - Assessment by injecting blank samples after a high concentration sample or calibration standard during method development.
    - If unavoidable:
      - Specific measures should be considered.
      - Tested during the validation.
      - Applied during the analysis of the study samples.
    - Injection of blank samples after samples with an expected high concentration, before the analysis of the next study sample.
    - Randomisation of samples should be avoided.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Calibration/Standard Curve**

    Same matrix as the samples in the intended study spiked with known concentrations (on basis of the concentration range expected).

    Number of standards: function of the anticipated range of analytical values, nature of the analyte/response relationship.
    - Blank sample (matrix sample processed without internal standard),
    - Zero sample (matrix sample processed with internal standard),
    - 6 – 8 non-zero samples covering the expected range, including LLOQ.

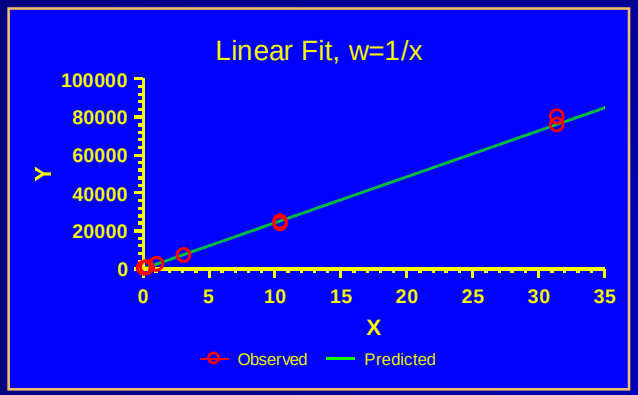
Pre-Study Validation

- **Full Validation** (cont'd)
  - **Calibration/Standard Curve** (cont'd)
    - Simplest model that adequately describes the concentration-response relationship should be used (*e.g.*, *F*-test, Minimum AIC).
    - Selection of weighting and use of a complex regression equation should be justified (analysis of residuals; *F*-test, Minimum AIC).
    - Response at LLOQ: ≥5 times response of blank.
    - Response at LLOQ: inprecision ≤20%, accuracy ±20% from nominal concentration.
    - Response at other levels: accuracy ±15% from nominal concentration.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Calibration/Standard Curve** (cont'd)
    - At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration.
    - Excluding individual standard points must not change the model used.

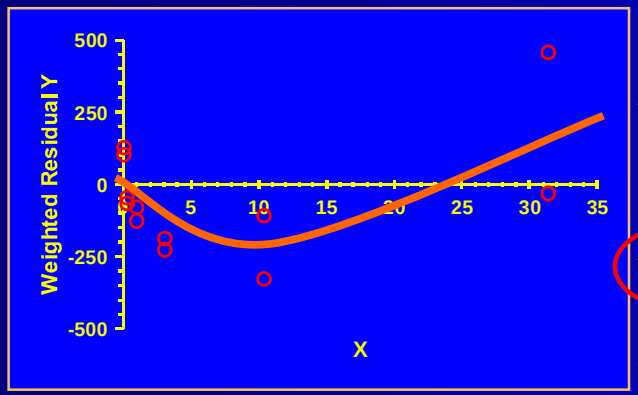
Case Study



Sum of squared residuals:
0.194707E+08

Sum of weighted squared residuals:
473468

S:
217.593 (df =10)

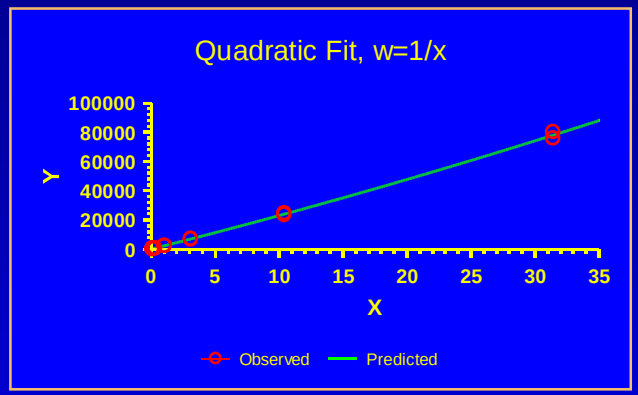


Correlation:
0.9992

Weighted correlation:
0.9989

AIC:
160.81407

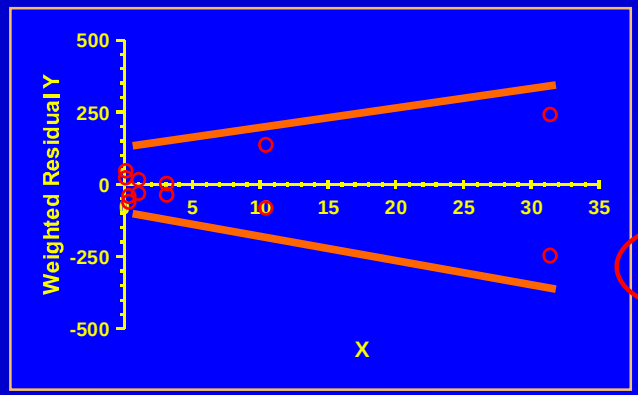
4 runs:
++--------+-



Sum of squared residuals:
0.965269E+07

Sum of weighted squared residuals:
155546

S:
131.465 (df=9)



Correlation:
0.9995

Weighted correlation:
0.9996

AIC:
149.45640

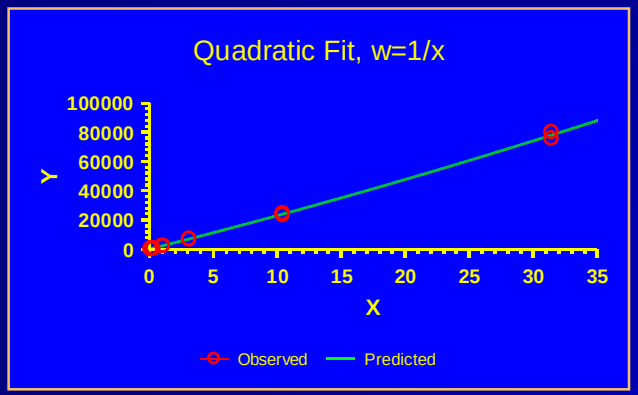
6 runs:
++---++--++-

Case Study

Back-calculated standards (linear, 1/x)			
nominal	Acc [%]	mean	CV [%]
0.102	125.09	122.7	2.77
0.102	120.29		
0.313	94.58	93.4	1.78
0.313	92.23		
1.045	91.95	93.4	2.23
1.045	94.90		
3.107	93.15	92.4	1.09
3.107	91.73		
10.42	93.47	95.6	3.21
10.42	97.81		
31.42	105.19	102.4	3.84
31.42	99.62		

Back-calculated standards (quadr., 1/x)			
nominal	Acc [%]	mean	CV [%]
0.102	109.96	107.3	3.46
0.102	104.70		
0.313	94.76	93.5	1.94
0.313	92.19		
1.045	97.81	99.4	2.28
1.045	101.02		
3.107	100.06	99.3	1.08
3.107	98.54		
10.42	98.31	100.5	3.10
10.42	102.72		
31.42	102.40	100.0	3.47
31.42	97.50		

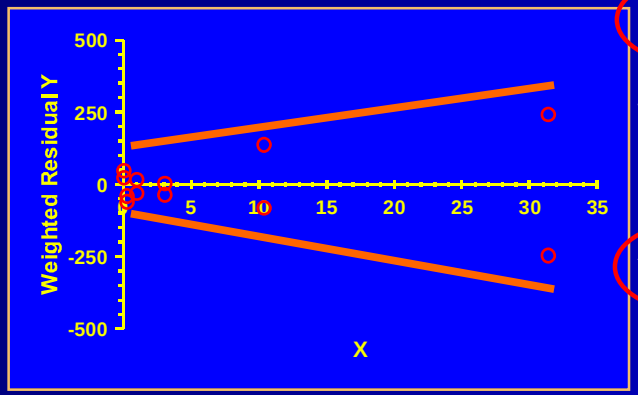
Case Study



Sum of squared residuals:
0.965269E+07

Sum of weighted squared residuals:
155546

S:
131.465 (df=9)

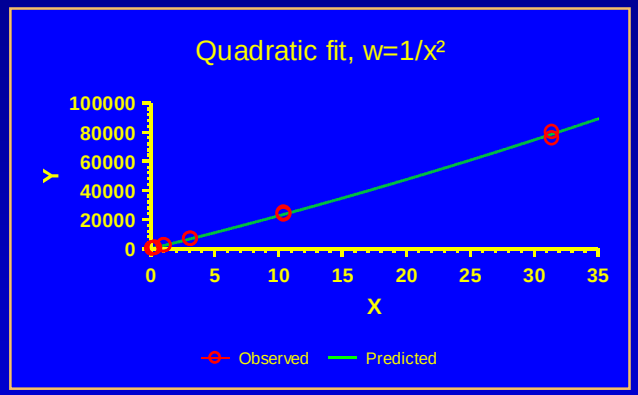


Correlation:
0.9995

Weighted correlation:
0.9996

AIC:
149.45640

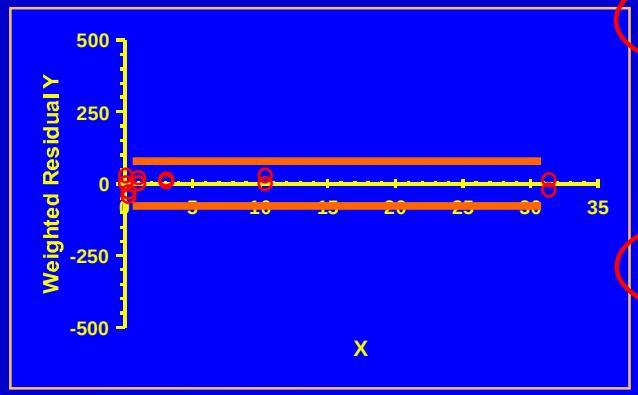
6 runs:
++---++--++-



Sum of squared residuals:
0.924405E+10

Sum of weighted squared residuals:
5751.13

S:
25.2787 (df =9)



Correlation:
0.9994

Weighted correlation:
0.9990

AIC:
109.88582

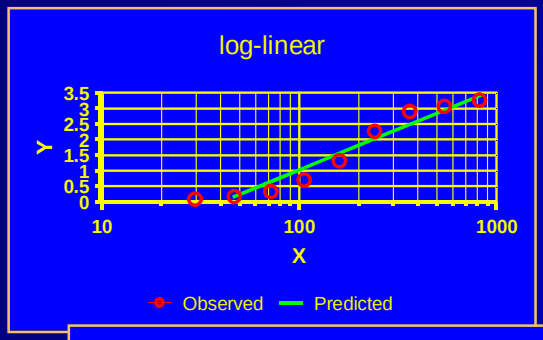
6 runs:
++--++++-++-

Case Study

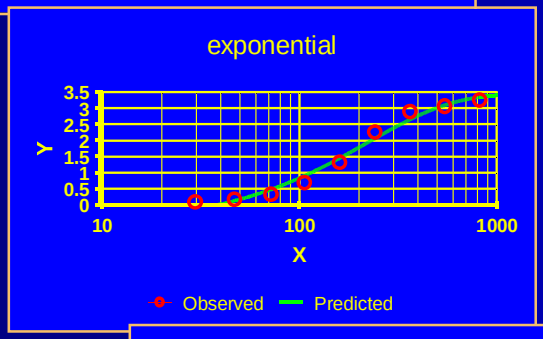
Back-calculated standards (quadr., 1/x)			
nominal	Acc [%]	mean	CV [%]
0.102	109.96	107.3	3.46
0.102	104.70		
0.313	94.76	93.5	1.94
0.313	92.19		
1.045	97.81	99.4	2.28
1.045	101.02		
3.107	100.06	99.3	1.08
3.107	98.54		
10.42	98.31	100.5	3.10
10.42	102.72		
31.42	102.40	100.0	3.47
31.42	97.50		

Back-calculated standards (quadr., $1/x^2$)			
nominal	Acc [%]	mean	CV [%]
0.102	105.60	102.9	3.74
0.102	100.16		
0.313	95.19	93.9	1.99
0.313	92.54		
1.045	100.01	101.7	2.29
1.045	103.31		
3.107	102.52	101.7	1.08
3.107	100.96		
10.42	99.91	102.1	3.06
10.42	104.33		
31.42	101.69	99.3	3.37
31.42	96.95		

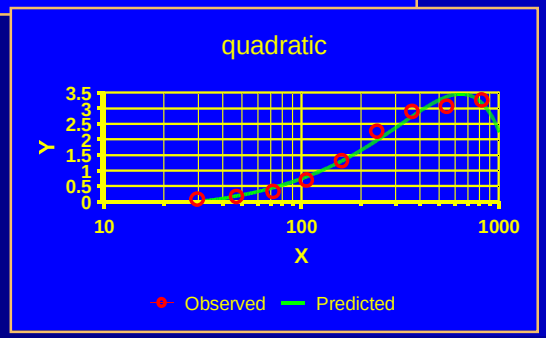
Example (LBA Calibration)



Correlation:
0.9715
AIC:
1.76539



Correlation:
0.9910
AIC:
-6.47417



Correlation:
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AIC:
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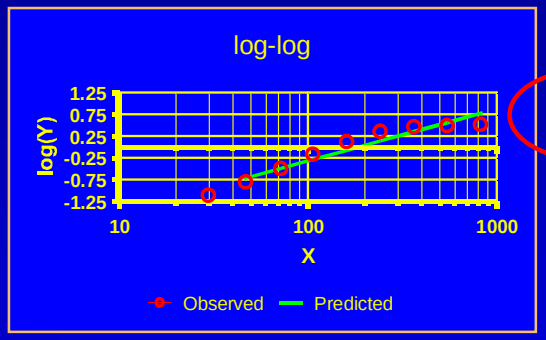
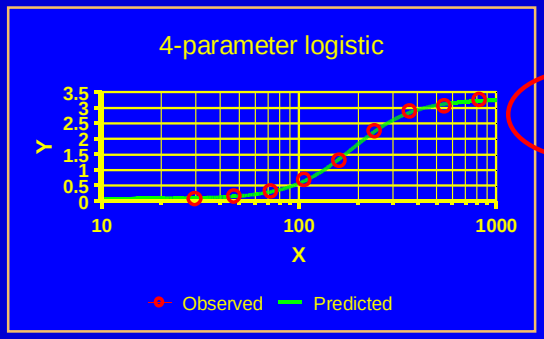


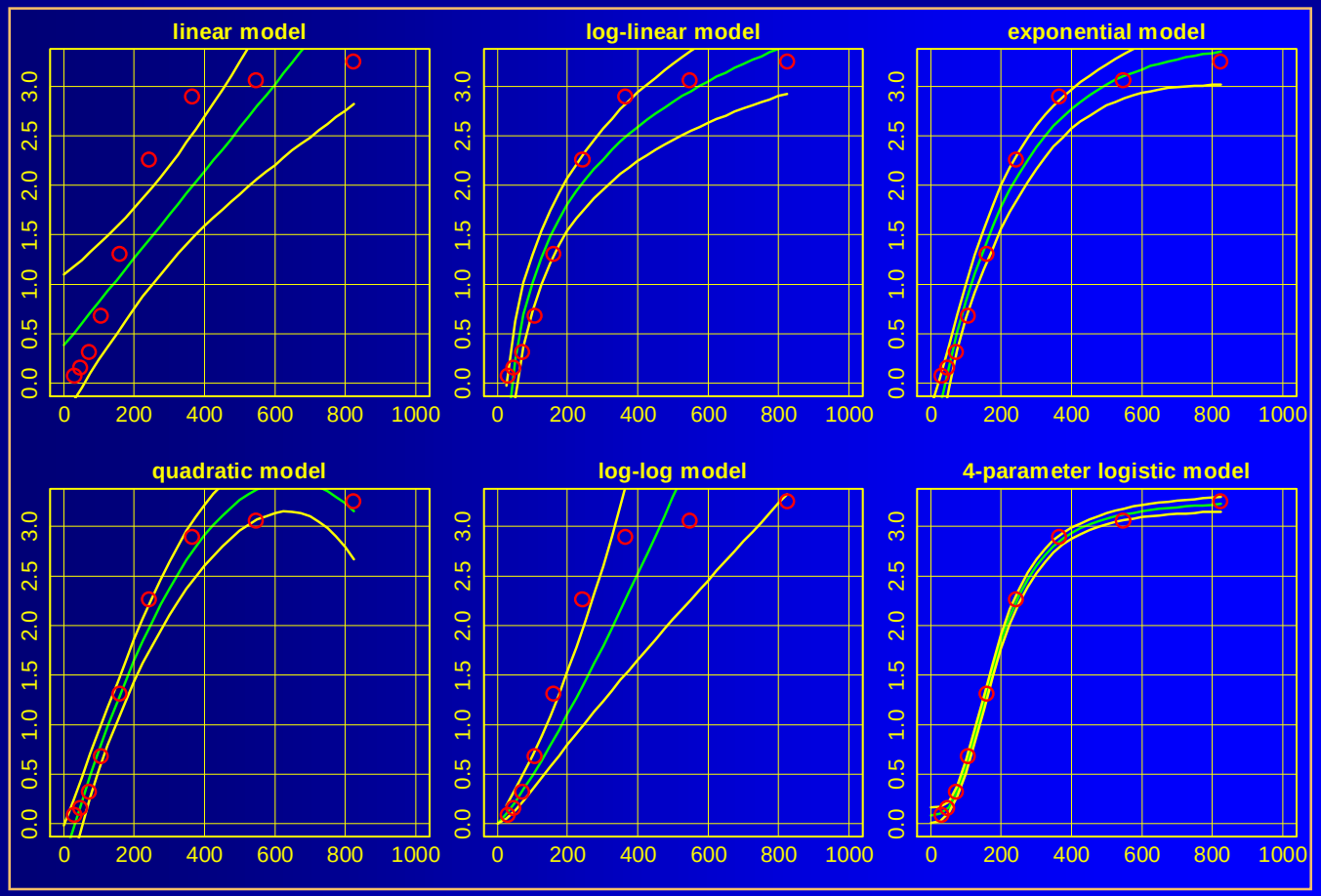
Correlation:
0.9624
AIC:
-9.68461



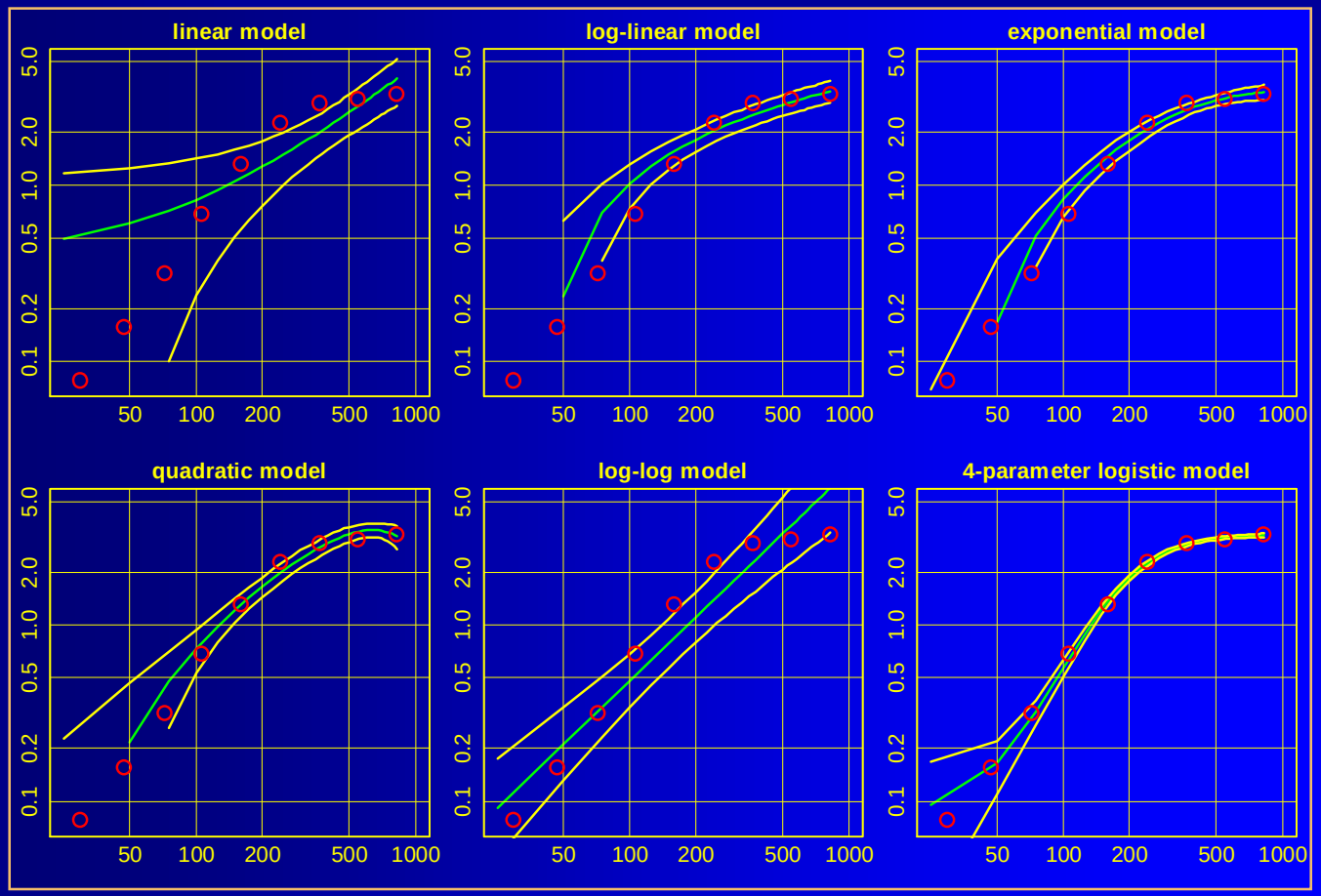
Correlation:
0.9996
AIC:
-31.69985

Example data (monoclonal antibody enzyme-linked immunosorbent assay) from Findlay & Dillard (2007)

Example (LBA Calibration)



Example (LBA Calibration)



Example (LBA Calibration)

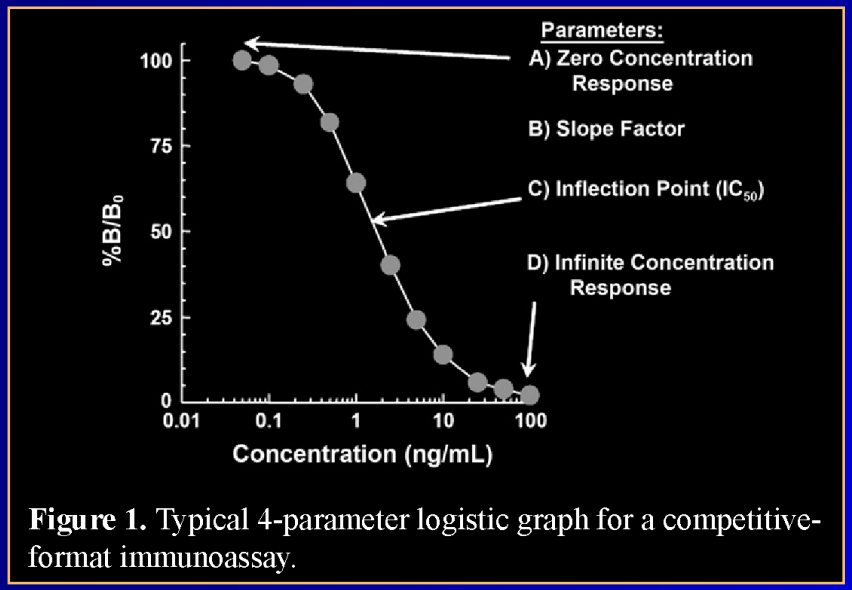


Figure 1. Typical 4-parameter logistic graph for a competitive-format immunoassay.

$$y = D + \frac{(A - D)}{\left(1 + \left(\frac{x}{C}\right)^{B}\right)}$$

LBA Calibration

- **Recommendations for 4-PL model**
  - **Optimal Assay Design for Calibration**
    - ≥5 calibration concentrations (according to Arlington III WP ≥6) and not more than 8.
    - Calibrators should be prepared and analyzed in duplicate or triplicate.
    - Concentration progression should be logarithmic, typically of the power of 2 or 3.
    - Midpoint concentration of calibrators should be somewhat greater than IC_{50}.
    - Anchor concentrations outside the expected validated range should be considered for inclusion to optimize the fit.
    - Suboptimal plate layouts should be avoided.

LBA Layout

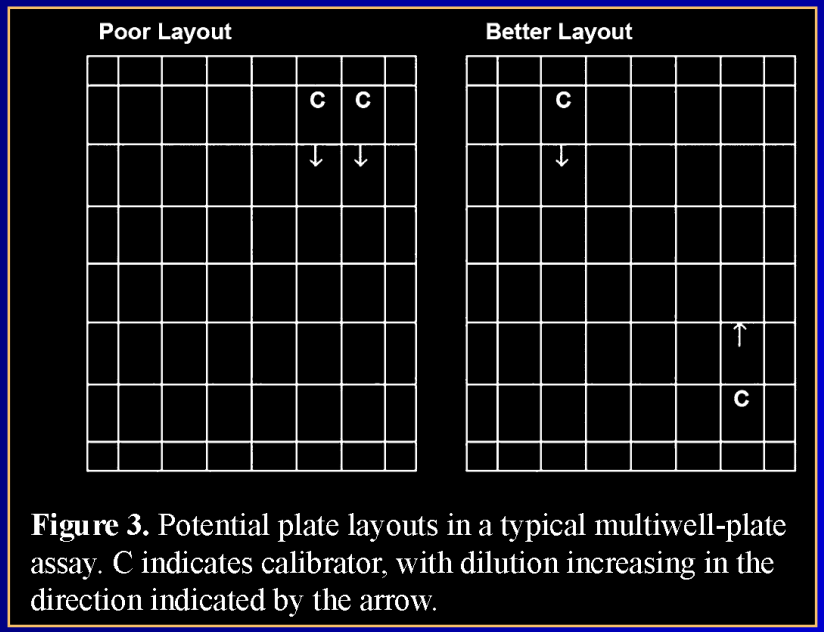


Figure 3. Potential plate layouts in a typical multiwell-plate assay. C indicates calibrator, with dilution increasing in the direction indicated by the arrow.

At left is a commonly used layout for an assay in which the calibrators are prepared in duplicate. In this plate configuration calibrators are always located in the same wells on the upper right of the plate. This layout helps to ensure proper identification of calibrators, but it is a scheme that is susceptible to positional effects on the plate.

The layout on the right is a much better choice. In this scheme the calibrators (as well as quality control [QC] samples and study samples) are distributed more widely on the plate, with one of the replicates positioned on the left side and the other on the right. The dilution direction is also reversed, with increasing dilution going down the plate on the left side and up the plate on the right.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Stability**

    Stability of the analytes during sample collection and handling.
    - Three Freeze-Thaw Cycles

      ≥3 aliquots at low and high levels stored for 24 hours and thawed unassisted (?!) at room temperature.
      When completely thawed, refrozen for 12 to 24 hours.
      This cycle two more times repeated, then analyzed after the third cycle.
      If instable: samples should be frozen at -70 ℃ dur ing another FT-cycle.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Stability** (cont'd)
    - Short-Term Storage (bench top, room temperature)
      Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Stability** (cont'd)
    - Long-Term Storage (frozen at the intended storage temperature) should exceed the time between the date of first sample collection and the date of last sample analysis.
      Determined by storing ≥3 aliquots of low/high levels under the same conditions as the study samples.
      Volume should be sufficient for analysis on three occasions. Concentrations of all samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Stability** (cont'd)
    - Long-Term Storage

      Often not finished when clinical phase already starts (Validation report contains a phrase like: *'long-term stability in progress'*). Not recommended, see http://www.emea.europa.eu/pdfs/human/chmptemplates/D80_AR_Generics_Non-Clinical_Clinical_Guidance.pdf, http://www.emea.europa.eu/Inspections/docs/gcp/INS-GCP-3a7.pdf

      Brief description of analytical methods used, with emphasis on the performance characteristics of assay validation and quality control. Provide information regarding where the bioanalysis was performed. In addition, it is essential to include the date of the start and finish of the bio-analytical phase to see if the long-term stability data of the pre-study validation is enough. Storage conditions of the samples should be stated.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Stability** (cont'd)
    - Stock Solution Stability of drug and the internal standard should be evaluated at room temperature for ≥6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.
      Arlington III WP: *If the reference standard is within its expiration date when the stock solution is prepared, there is no need to prepare a new stock solution when the reference standard expires.*

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Stability** (cont'd)
    - Post-Preparative Stability

      Stability of processed samples, including the resident time in the autosampler, should be determined.

      The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Pre-Study Validation

- **Full Validation** (cont'd)
  - **Sample dilutions**
    of concentrations above the ULOQ.
    - *E.g.*, ~140 % of ULOQ diluted 1:1.
    - Blank matrix should be used in dilution.
    - Replicate (≥5) analysis.
      - Imprecision (CV%): ≤15%
      - Inaccuracy (absolute mean bias – RE%): ≤15%

Pre-Study Validation

- **Partial Validation** (cont'd)
  - Method transfers between laboratories (or analysts…).
  - Change in analytical methodology (*e.g.*, change in detection systems).
  - Change in anticoagulant in harvesting biological fluid.
  - Change in matrix within species (*e.g.*, human plasma to human urine).
  - Change in sample processing procedures.

Pre-Study Validation

- **Partial Validation** (cont'd)
  - Change in species within matrix (*e.g.*, rat plasma to mouse plasma).
  - Change in relevant concentration range.
  - Changes in instruments and/or software platforms.
  - Limited sample volume (*e.g.*, paediatric study).
  - Rare matrices.
  - Selectivity demonstration of an analyte in the presence of concomitant medications and/or specific metabolites.

Performing the Validation

- Conducting the Validation strictly according to the Validation Plan!
  - Results must comply with limits set in the Validation Plan.
  - If not: The method is validated, but not valid!
- Report of Results:
  - Method Validation Report;
  - will be referred in the Analytical Protocol of PK/BA/BE-studies.

In-Study Validation

- Application of Validated Method to Routine Analysis
  - System Suitability (SS)
    - FDA (2001): Based on the analyte and technique, a specific SOP (or sample) should be identified to ensure optimum operation of the system used.
    - Arlington III (2007): As part of qualifying instruments, performance of SS ensures that the system is operating properly at the time of analysis.
      - SS checks are more appropriately used for chromatographic methods to ensure that the system is sufficiently sensitive, specific, and reproducible for the current analytical run.
      - However, the SS tests do not replace the required run acceptance criteria with calibration standards and QC samples.
      - SS tests, when appropriate, are recommended to ensure success, but are not required, nor do they replace the usual run acceptance criteria.

In-Study Validation

- Study Samples should be analyzed according to the Analytical Protocol.
  - Minimum number of QCs (in multiples of three) should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.
    - Low / intermediate / high concentration levels
      At least duplicates at each level.

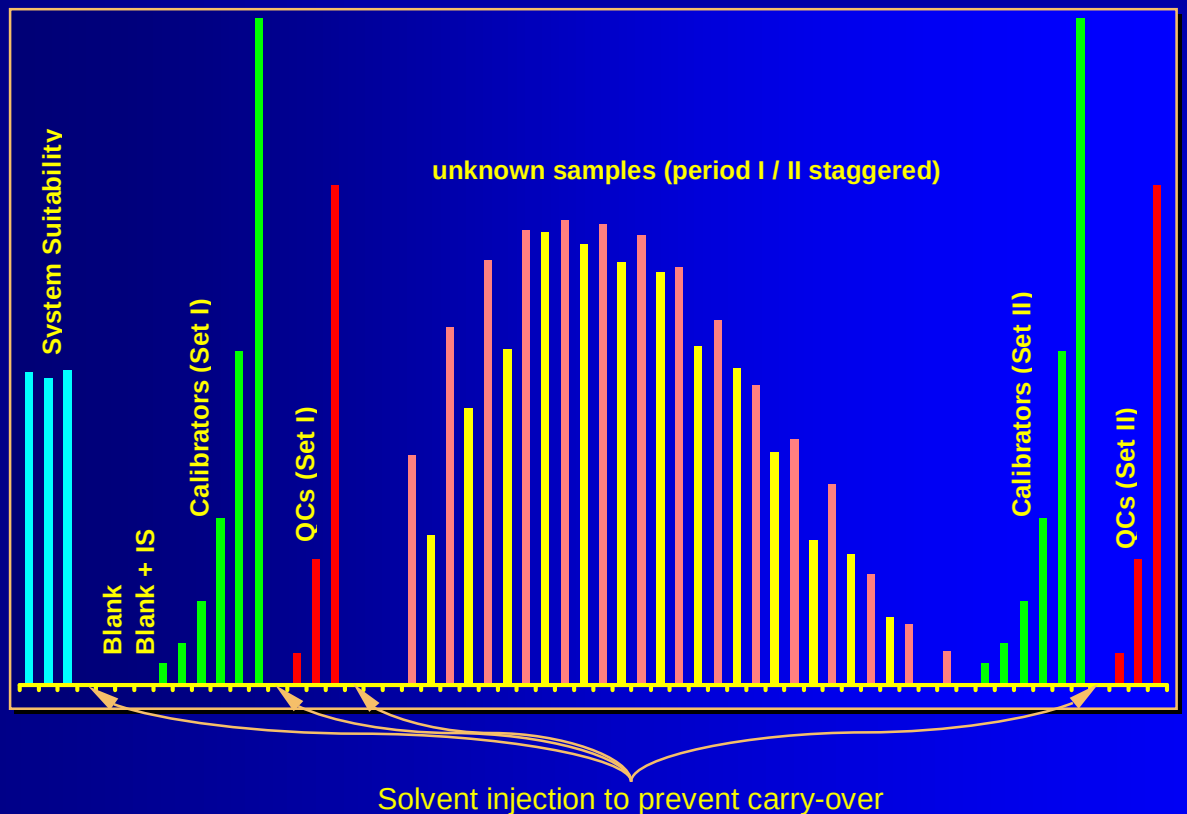
Low	within ≥LLOQ and 3×LLOQ
Intermediate	near the center of the calibration range ('center' according to Arlington III WP: geometric mean of LLOQ and ULOQ)
High	near the ULOQ (≥75% ULOQ)

In-Study Validation

- Study Analyses (cont'd)
  - Standards and QC samples can be prepared from the same spiking stock solution, provided the solution stability and accuracy have been verified (FDA 2001).
    - Some kind of a vicious circle:
      - You can use the same stock solution for calibration and QC samples.
      - You have to demonstrate that the stock solution was prepared correctly and that its concentration is accurate.
      - How can you do that without comparing it to another, independently prepared stock solution?

In-Study Validation

- Study Analyses (cont'd)



Typical Batch

Don't inject unknown samples in random order (carry-over)!

In-Study Validation

- Study Analyses (cont'd)
  - Quality Control Samples (QCs) should be analyzed together with Calibrators and study samples.
    - Acceptance Criteria for an analytical run

      QCs

      85% – 115% accuracy for single determinations of QCs; not more than 33% (two *different* out of six) per run should be out of range.

      Standard Curve

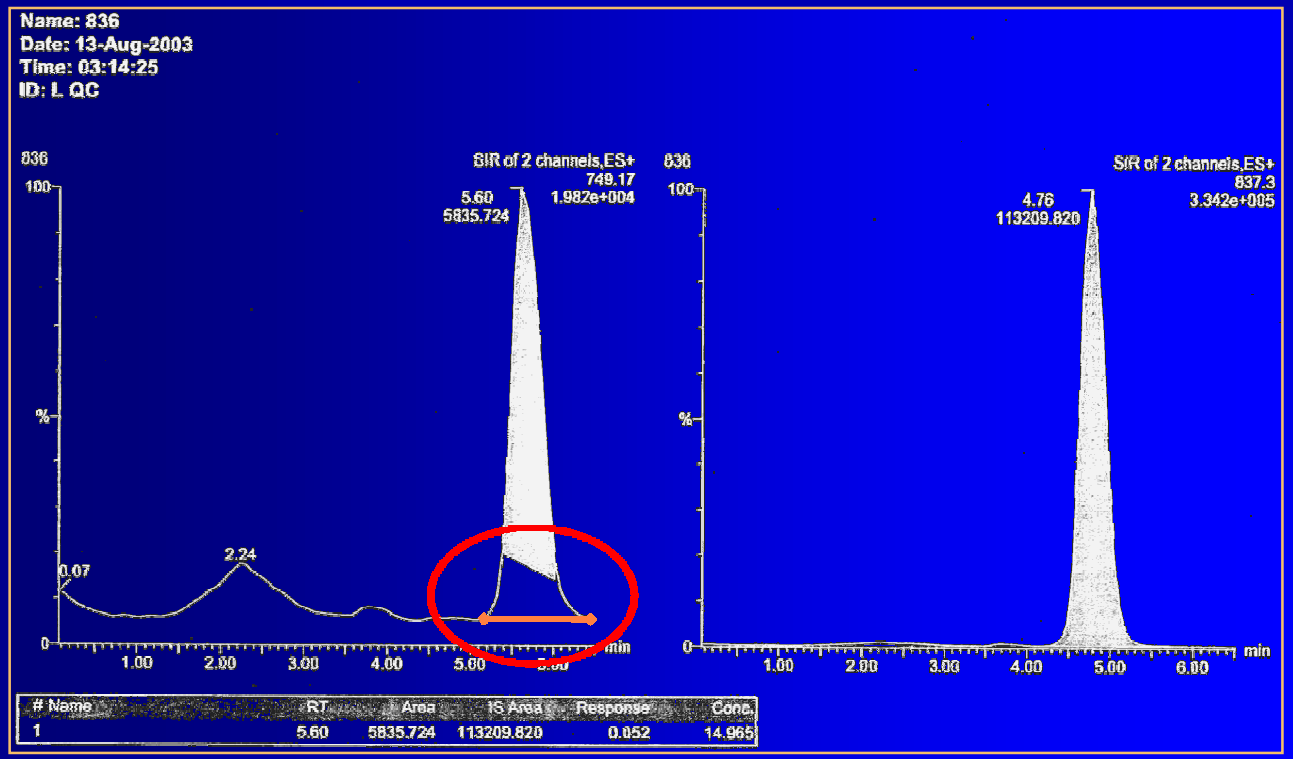
      85% – 115% accuracy for 75% of standard points, except at LLOQ (80% – 120%).

      Values outside this ranges can be discarded, provided they do not change the model established in validation.

      No rejection of calibrators based on results of QCs!

In-Study Validation

- Study Analyses (cont'd)
  - Do not try to fool inspectors!



In-Study Validation

- Study Analyses (cont'd)
  - Samples can be analyzed with a single determination […] if the assay method has acceptable variability as defined by validation data.
  - For a difficult procedure with a labile analyte*) where high precision and accuracy specifications may be difficult to achieve, duplicate or even triplicate analyses can be performed for a better estimate of analyte.

*) removed in Arlington III

Precision almost halved if going from singlets to triplicates!

replication	CV [%]				
0 (single)	20.0%	25.0%	30.0%	40.0%	50.0%
1 (duplicate)	14.1%	17.7%	21.2%	28.3%	35.4%
2 (triplicate)	11.5%	14.4%	17.3%	23.1%	28.9%
3 (quadruplicate)	10.0%	12.5%	15.0%	20.0%	25.0%

In-Study Validation

- Study Analyses (cont'd)
  - Acc. to Arlington III WP:
    - Mandatory SOPs (additional to the 'common' ones…):
      - Reintegration (incl. audit trail),
      - Reassay criteria.
  - EMA Draft GL:
    - Number of reanalysed samples (and % of total number of samples) should be discussed in the study report. Samples should be identified and the initial value, the reason for reanalysis, the values obtained in the reanalyses, the finally accepted value and a justification for the acceptance should be provided.

In-Study Validation

- Study Analyses (cont'd)
  - EMA Draft GL (cont'd):
    - Normally reanalysis of study samples because of a pharmacokinetic reason is not acceptable. This is especially important for bioequivalence studies, as this may affect and bias the outcome of such a study. However reanalysis might be considered as part of laboratory investigations, to identify possible reasons for results considered as abnormal and to prevent the recurrence of similar problems in the future.
  - EMA BE GL (Section 4.1.7, 2010)
    - First two sentences above stated in Section 4.1.7 Bioanalytical methodology.

In-Study Validation

- Repeated samples
  - SOP or guideline including acceptance criteria must be established explaining the reasons for repeating sample analysis.

    Reasons for repeat analyses could include:
    - repeat analysis of clinical or preclinical samples for regulatory purposes
    - inconsistent replicate analysis
    - samples outside of the assay range
    - sample processing errors
    - equipment failure
    - poor chromatography
    - inconsistent pharmacokinetic data (EMA: not acceptable!)

In-Study Validation

- Repeated samples (cont'd)
  - Reassays should be done in triplicate if sample volume allows.
    The rationale for the repeat analysis and the reporting of the repeat analysis should be clearly documented.
  - Currently no specific guidelines, but all repeated samples must be reported (original value, repeated value(s), used value, justification):
    - EU (Day 80 Critical Assessment Report, Generic medicinal product, 2006):
      http://www.emea.europa.eu/pdfs/human/chmptemplates/D80_AR_Generics_Non-Clinical_Clinical_Guidance.pdf
      Reasons for any reanalysis of samples and if the final value has been decided correctly according to the relevant SOP.

In-Study Validation

- Repeated samples (cont'd)
  - FAD/CDER/OGD (Jan 2007): http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/AbbreviatedNewDrugApplicationANDAGenerics/UCM120957.pdf

Table 9 Reanalysis of Study Samples

Study No. Additional information in Volume(s), Page(s)								
Reason why assay was repeated	Number of samples reanalyzed				Number of recalculated values used after reanalysis			
	Actual number		% of total assays		Actual number		% of total assays	
	T	R	T	R	T	R	T	R
Pharmacokinetic[1]								
Reason A (e.g. below LOQ)								
Reason B								
Reason C								
Etc.								
Total								

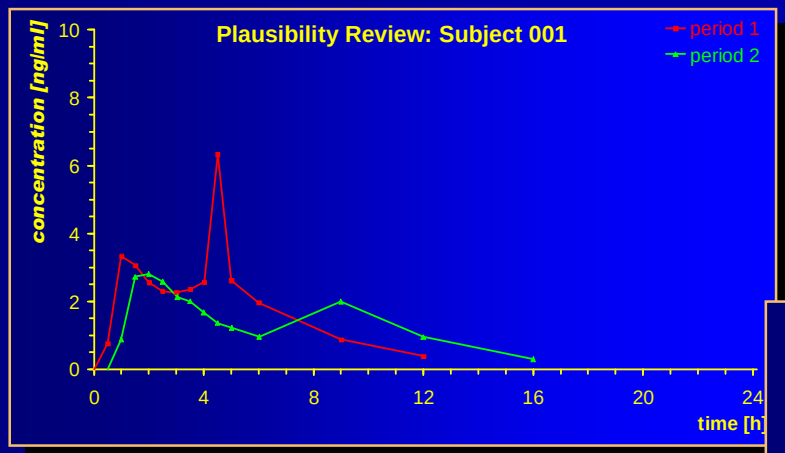
1 - If no repeats were performed for pharmacokinetic reasons, insert "0.0."

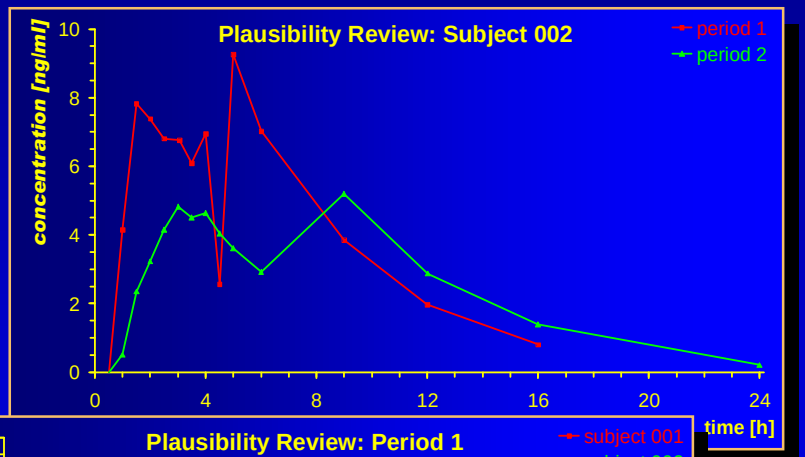
Please provide a separate table for each analyte measured for each in-vivo study.

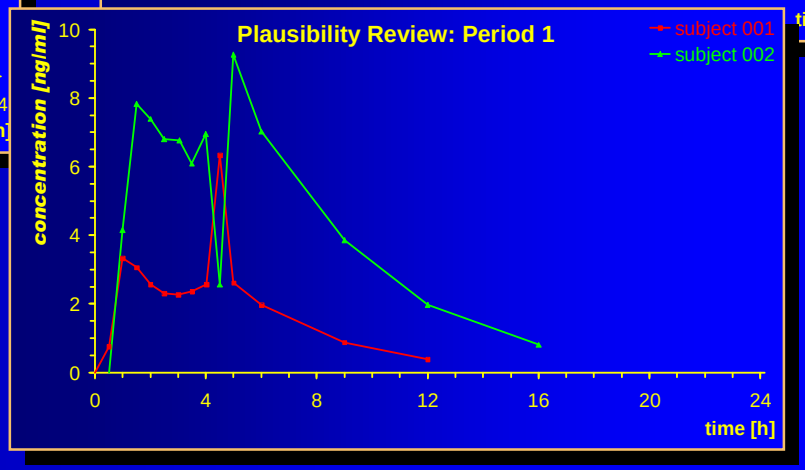
Plausibility Review

- Plausibility Review of analytical data
  - If ever possible, plan a *blinded* (**!**) Plausibility Review of analytical data by an independent Pharmacokineticist as early as possible.
  - QC-cleared data only; start of review earliest if analyses of ~50% of subjects are completed.
    - Consistency within subjects!
    - Pre-dose concentrations?
    - Rising values in the terminal phase?
    - Fluctuating values at C_{max}?
    - Re-analysis ('pharmacokinetic repeats'): values confirmed/rejected?
    - Reanalysis of study samples because of a PK reason is not acceptable (EMA BE GL 2010).

Case Study





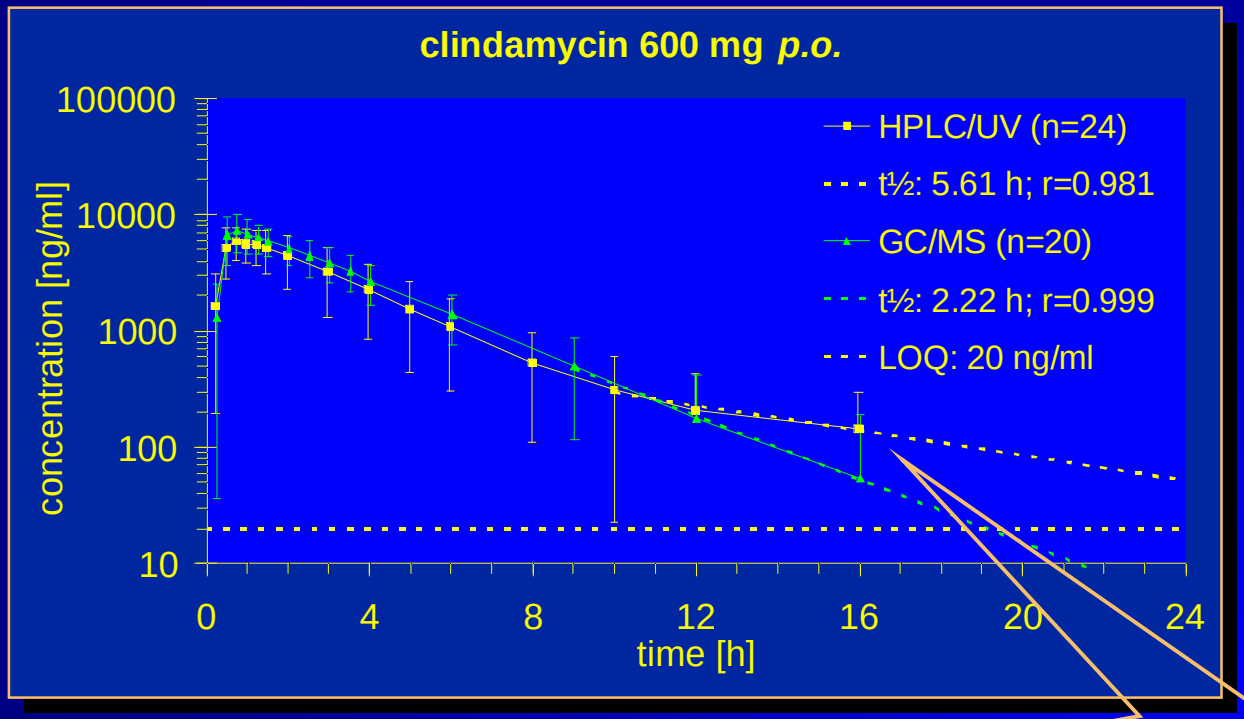


subject	time [h]	analyte [ng/ml]	γ-GT [U/l]	albumine [g/dl]
001	4.0	2.572	13	3.8
001	4.5	6.330	9	3.5
001	5.0	2.615	14	3.9
002	4.0	6.956	9	3.4
002	4.5	2.561	14	4.0
002	5.0	9.262	8	3.4

Cross-Validation

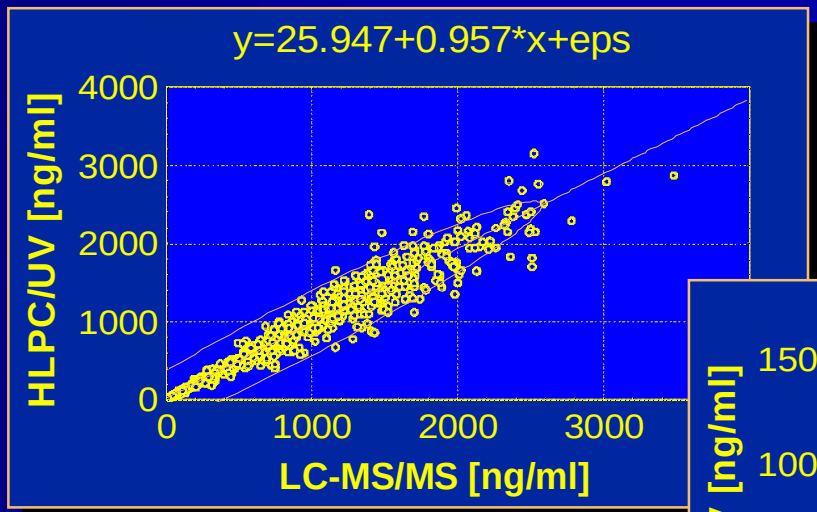
- Comparison of validation parameters when ≥2 analytical methods are used to generate data within the same study or across different studies. Example: an original validated bioanalytical method serves as the *reference* and a revised bioanalytical method is the *comparator*.
- Cross-validation should also be considered when data generated using different analytical techniques (*e.g.*, LC/MS-MS *vs.* ELISA) in different studies are included in a regulatory submission.
- No specific recommendations in Arlington III WP.

Case Study

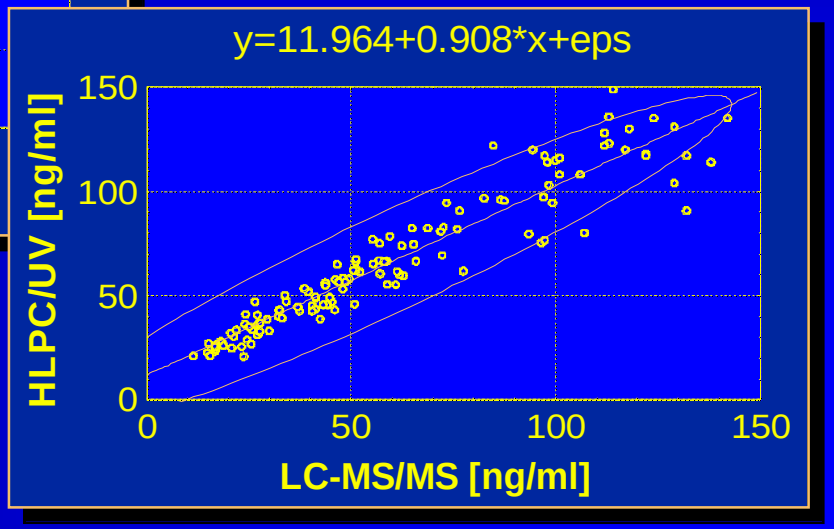


Identical LLOQ, but bias in lower concentration range?

Case Study



Bias in lower concentration range!



Reporting Results

- Avoid discrepancies between electronic data and paper reports.
  - Problems arise if electronic data in full precision are transferred to the statistical database.
  - Generally (paper-)reports contain only modified results (rounded to decimal places or significant figures, or – even worse – truncated values).
  - If PK-parameters have to be re-calculated from the paper-version or a PDF-file (*i.e.*, during an inspection), results may differ from ones reported…

Reporting Results

- Reasons for rounding of analytical data:
  - Pragmatic: avoid discrepancies between paper and electronic data which may raise unnecessary questions.
  - Scientific: use of full precision data implies a degree of accuracy/precision which is illusionary.

Raw data	3 decimal places	3 significant figures
31.41592653589793	31.416	31.4
3.14159265358979	3.142	3.14
0.31415926535898	0.314	0.314

Rounding to three decimal places is suggesting an ability to distinguish between 31.4154 and 31.4165 – a difference of 0.0035 % from the reported value!

Better, but implies still ~0.2% precision!

Reporting Results

- Personal opinion:
  - Most analysts have digested Arlington I-II and are familiar with 15 % accuracy / precison (20 % at LLOQ), but routinely come up with results like
    3.141 592 653 589 793.*)
  - Subconsciously they *belief*, that such a result *is more correct* than *3.14*.
  - If suggesting next time they should come up with
    3.141 592 653 589 793 238 462 643 383 279 502 88,
    they tell me, that I am a funny person…

*) at 15 % CV: 95 % Confidence Interval **[2.21 – 4.07]**
at 5 % CV: 95 % Confidence Interval **[2.83 – 3.45]**

Open Issue

I have no opinion about *'incurred samples'* – an expression which has no easily understandable meaning for me in the English language.

Nick Holford

http://www.boomer.org/pkin/PK07/PK2007010.html

Open Issue

- Incurred Sample Re-Analysis (Arlington III)
  - Situations, where standards and QCs may not adequately mimic that of study samples form dosed subjects.
    - Metabolites converting to parent compound,
    - Proteinbinding differences in patient samples,
    - Recovery issues,
    - Sample inhomogeneity,
    - Mass spectrometric ionization matrix effects.
  - It is generally accepted that the chance of incurred sample variability is greater in humans than in animals, so the following discussion pertains primarily to clinical studies.
    - Final decision as to the extent and nature of the incurred sample testing is left to the analytical investigator, and should be based on an in-depth understanding of the method, the behavior of the drug, metabolites, and any concomitant medications in the matrices of interest.

Open Issue

- Incurred Sample Re-Analysis (cont'd)
  - Considerations in selecting samples to be reassayed:
    - concentration,
    - patient population, and
    - special populations (*e.g.*, renally impaired),
    - depending on what is known
      - about the drug,
      - its metabolism,
      - and its clearance.
  - Examples of studies that should be considered for incurred-sample concentration verification are
    - First-in-human,
    - Proof-of-concept in patients,
    - Special population, and
    - Bioequivalence (!) studies.

Open Issue

- Incurred Sample Re-Analysis (cont'd)
  - Re-assay of 15% of samples was required in Canada since 1992, but was removed in Sep 2003.
  - Health Canada on 09 Jan 2008 published a 'Notice: Replication of Incurred Samples in Bioavailability/Bioequivalence Studies':
    - '[…] a voluntary submission of data collected on replicate samples since 2000. […] This information will be used for research purposes only and will in no way affect past regulatory decisions. [...] Release of the information will be limited to summary statistics, with no linkage between the sponsor and the data.'
    - HPB hopes '… to be able to present our findings at the next Canadian Workshop on Recent Issues in GLP Bioanalysis on April 17-18, 2008 in Montreal.'

http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/prodpharma/notice_bioan_avis_anbio_e.pdf

Open Issue

- Incurred Sample Re-Analysis (cont'd)
  - European Initiative started by the 'European Bioanalysis Forum':
    http://www.aapspharmaceutica.com/meetings/files/112/PhilipTimmermanebfperspective.pdf
    Until now only open to the industry, but collaboration planned with other scientific and interprofessional groups on BA related topics (academia, vendors, CROs, or regulatory bodies)…
  - AAPS Workshop on Current Topics in GLP Bioanalysis: Assay Reproducibility for Incurred Samples Samples – Implications of Crystal City Recommendations (Feb 2008)
    http://www.aapspharmaceutica.org/meetings/meeting.asp?id=112
    http://www.aapspharmaceutica.org/GLP/
  - EBF/EUFEPS Workshop (Apr 2010)
    http://www.eufeps.org/spring10041516.html

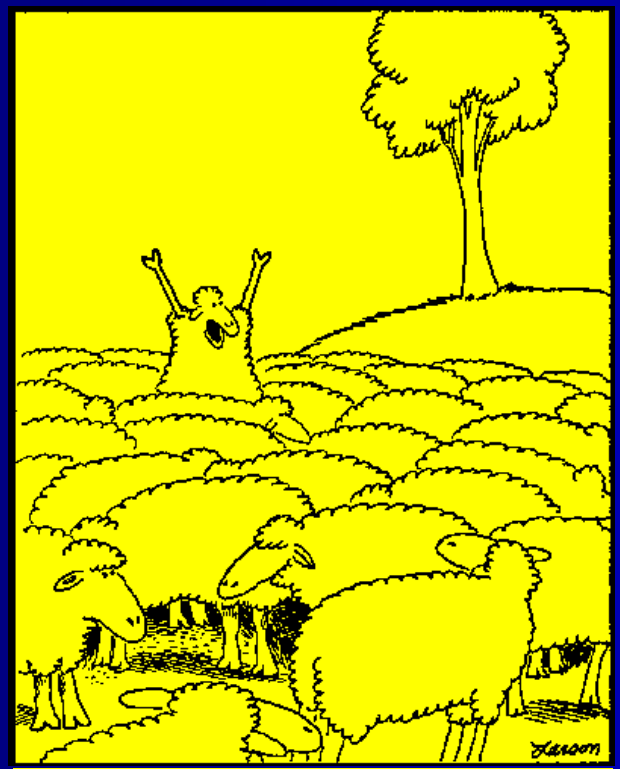
Open Issue

- Incurred Sample Re-Analysis
  - Expected to be mandatory for the FDA.
  - Will not be necessary in Canada.
  - EMA: ‘For BE studies analysis of incurred samples should always be carried out.’ (see below)
- EMA GL on Validation of Bioanalytical Methods (Finalization in 2010?)
  - Concept Paper/Recommendations on the Need for a (CHMP) Guideline on the Validation of Bioanalytical Methods (12/2008) http://www.emea.europa.eu/pdfs/human/ewp/53130508en.pdf
  - Draft GL on the Validation of Bioanalytical Methods (11/2009, Deadline for Comments 31 May 2010) http://www.ema.europa.eu/pdfs/human/ewp/19221709en.pdf

Open Issue

- Incurred Sample Re-Analysis (EMA Draft 2009, Section 6)
  - Recommended to evaluate accuracy of incurred samples by reanalysis of study samples over a certain time period. Extent of testing depends on the analyte and the study samples, and should be based upon in-depth understanding of the analytical method and analyte. Should provide sufficient confidence that the concentration being reported is accurate.
  - For BE studies ICSR should always be carried out.
  - Evaluated as early as possible.
  - From several subjects around Cmax and in the elimination phase.
  - Difference between two results should be ≤20% of the mean (≤30% for LBAs) for ≥⅔ of repeats.

Outlook



**“Wait! Wait! Listen to me! …
We don’t HAVE to be just sheep!”**

- David Bourne’s (Uni. Oklahoma) e-mail list
  - A rather active list (3200+ members, about 50 postings/week) covering almost any aspect of PK/PD/bioanalytics…
    - Subscription http://www.boomer.org/pkin/
    - Search page http://www.boomer.org/pkin/simple.html
- BA and BE Forum (BEBAC Vienna)
  - Specialized in BA/BE/bioanalytics.
    - No registration necessary to *read* posts. http://forum.bebac.at/
    - Registration (to post): http://forum.bebac.at/register.php

Thank You! Hvala!
Analytical Development and Validation
Open Questions?

(References and a Summary of the 'Arlington III White Paper' in your Handouts)

Helmut Schütz
BEBAC
Consultancy Services for
Bioequivalence and Bioavailability Studies
1070 Vienna, Austria
helmut.schuetz@bebac.at

References

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  - Q2B: Validation of Analytical Methods: Methodology (1996)
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- WHO
  - Handbook for GLP (2001)
  - Fortieth Report - TRS No. 937 (2006)
    - Annex 7: Multisource (generic) pharmaceutical products: guidelines on registration requirements to establish interchangeability
    - Annex 9: Additional guidance for organizations performing *in vivo* bioequivalence studies
- US-FDA
  - Center for Drug Evaluation and Research (CDER) Reviewer Guidance: Validation of Chromatographic Methods (Nov 1994)
  - Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) Guidance for Industry. Bioanalytical Method Validation (May 2001)
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  - Manual for Good Bioavailability and Bioequivalence Studies.
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  - Validation of Analytical Procedures (Jun 2005)
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    Part II – other than compliance testing
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    Annex 2: Qualification of GC Equipment (Oct 2006)

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The AAPS Journal 9(2) Article 29, E260-E267 (2007)
http://www.aapsj.org/articles/aapsj0902/aapsj0902029/aapsj0902029.pdf

Routine Drug Analysis Process and Run Acceptance Criteria (Arlington III WP)

Process or Criteria	Chromatographic Assays	Ligand-Binding Assays
Preparation of standards and QC samples	Standards and QC samples can be prepared from the same spiking stock solution, provided the solution stability and accuracy have been verified. A single source of matrix may also be used, provided selectivity has been verified.	
Placement of samples	Standard curve samples, blanks, QCs, and study samples can be arranged as considered appropriate within the run, and support detection of assay drift over the run.	
Number of calibration standards in a run	Include with each analytical batch: ■ Blank matrix (sample without IS) ■ Zero standard (matrix sample with IS) ■ Non-zero calibration standards: ≥6 standard points	Include with each analytical batch or microtiter plate: ■ Blank matrix ■ Non-zero calibration standards: ≥6 standard points. Can include anchor points (below LLOQ or above ULOQ in the asymptotic low- and high-concentration end of the standard curve).

Routine Drug Analysis Process and Run Acceptance Criteria (Arlington III WP)

Process or Criteria	Chromatographic Assays	Ligand-Binding Assays
Acceptance criteria for calibration standards	Residuals (absolute difference between the back calculated and nominal concentration) for each calibration standard should meet the following limits: ■ LLOQ standard <20% ■ All other standards <15%	Residuals for each calibration standard should meet the following limits: ■ LLOQ and ULOQ standards <25% ■ All other standards <20% ■ Any anchor points if used, are not to be included in the above acceptance criteria.
	A minimum of 75% standards (at least 6 nonzero points) should be within the above limits for the analytical run to qualify. Values falling outside these limits can be discarded, provided they do not change the established model.	

Routine Drug Analysis Process and Run Acceptance Criteria (Arlington III WP)

Process or Criteria	Chromatographic Assays	Ligand-Binding Assays
Number of QC samples in a batch	Include QC samples at the following 3 concentrations (within the calibration range) in duplicate with each analytical batch: ■ Low: near the LLOQ (up to 3× LLOQ) ■ Medium: midrange of calibration curve ■ High: near the high end of range	QC samples at the following 3 concentrations (within the calibration range) in duplicate should be added to each microtiter plate: ■ Low: above the second non-anchor standard, ~3× LLOQ ■ Medium: midrange of calibration curve ■ High: below the second non-anchor point high standard at ~75% of ULOQ
	Each analytical batch should contain 6 or a minimum of 5% of the total number of unknown samples. Add QCs in multiples of three concentrations (low, medium, high) when needed.	

Routine Drug Analysis Process and Run Acceptance Criteria (Arlington III WP)

Process or Criteria	Chromatographic Assays	Ligand-Binding Assays
Acceptance criteria for QC samples	Allowed % deviation from nominal values: ■ QCs prepared at all concentrations greater than LLOQ <15% ■ Low QC (if prepared at LLOQ) <20%	Allowed % deviation from nominal values: ■ QCs prepared at all concentrations other than LLOQ and ULOQ <20 % ■ Low and high QC (if prepared at LLOQ or ULOQ) <25% ■ In certain situations wider acceptance criteria may be justified, *e.g.*, when total error during assay validation approaches 30%.
	At least 67% (4 of 6) of the QC samples should be within the above limits; 33% of the QC samples (not all replicates at the same concentration) can be outside the limits. If there are more than 2 QC samples at a concentration, then 50% of QC samples at each concentration should pass the above limits of deviation.	

Routine Drug Analysis Process and Run Acceptance Criteria (Arlington III WP)

Process or Criteria	Chromatographic Assays	Ligand-Binding Assays
Replicate analysis	In general, samples can be analyzed with a single determination without replicate analysis if the assay method has acceptable variability as defined by the validation data. Duplicate or replicate analysis can be performed for a difficult procedure where high precision and accuracy may be difficult to obtain.	Accuracy can generally be improved by replicate analysis. Therefore, duplicate analysis is recommended. If replicate analysis is performed, the same procedure should be used for samples and standards.
Multiple analytes in a run	Samples involving multiple analytes in a run should not be rejected based on the data from one analyte failing the acceptance criteria.	
Rejected runs	The data from rejected runs need not be documented, but the fact that a run was rejected and the reason for failure should be reported.	