Limitations of current PK assay guidelines for the validation of active drug assays

Eginhard Schick

Large Molecule Bioanalytical R&D, pRED Pharmaceutical Sciences

Roche Innovation Center Basel, Switzerland

EBF – Focus Workshop, 21-22 June 2017, Lisbon

Bioanalytical Strategies for Large Molecules in Modern Drug Development: LBA and LC-MS United

Outline

- **Introduction**
  - Why active protein drug quantification matters ...
  - Requirements for active drug quantification
  - Assay concept for active drug quantification
- **Active drug assays and current guidelines**
  - Validation concept for active drug assays
  - Calibration concept, free analyte QC concept
  - Assessment of validation parameters
  - Challenges/limitations with regards to current guidelines
- **Summary and conclusions**

Active Drug Quantification

Requirements for Active Drug Quantification

- The validity of a non-clinical safety study relies upon the demonstration of **active drug exposure** throughout the dosing phase of the study

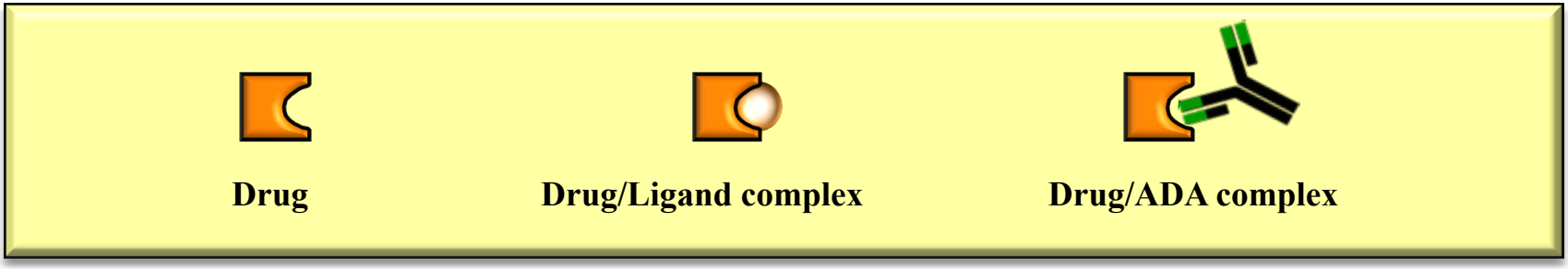
  Ponce et al., Regul. Toxicol. Pharmacol. 54(2), 164-182 (2009)

- The **interference** of **soluble targets** (or extracellular domain of the target receptor), or **ADA** on the PK assay need to be considered in the design of the sampling strategy by **looking for active or total drug**

  Chirmule et al., AAPS. J. 14(2), 296-302 (2012)

- The success of the PK–PD modeling effort depends on close communication between the PK–PD bioanalytical and clinical scientists: **The PK–PD model is only as good as the data provided for modeling**

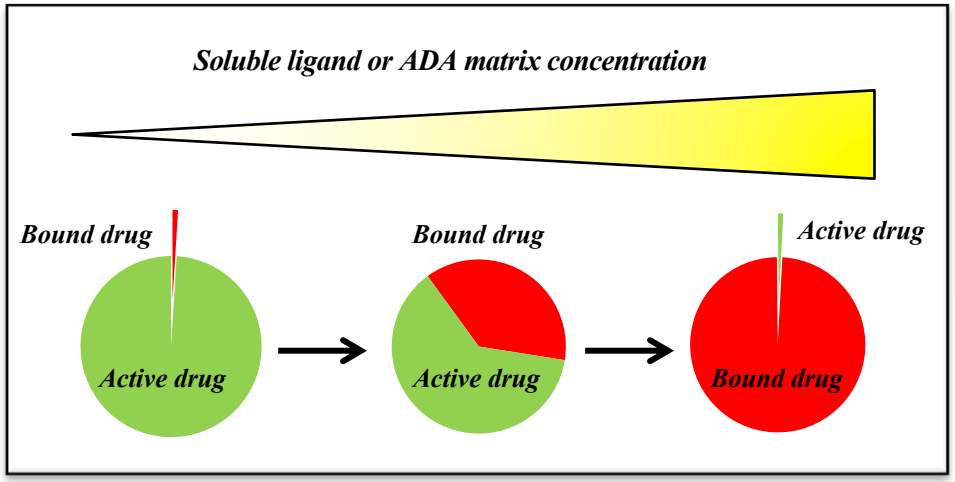
  Roskos et al., Bioanalysis. 3(6), 659-675 (2011)

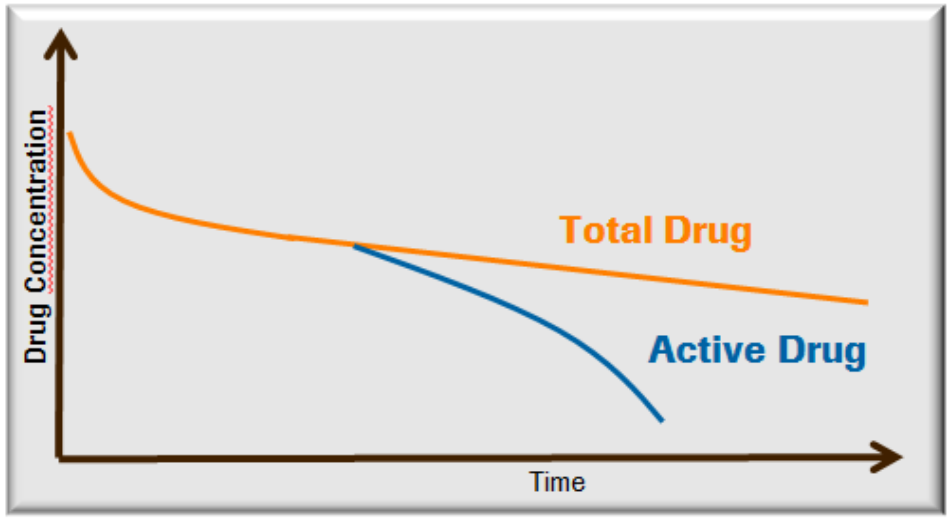


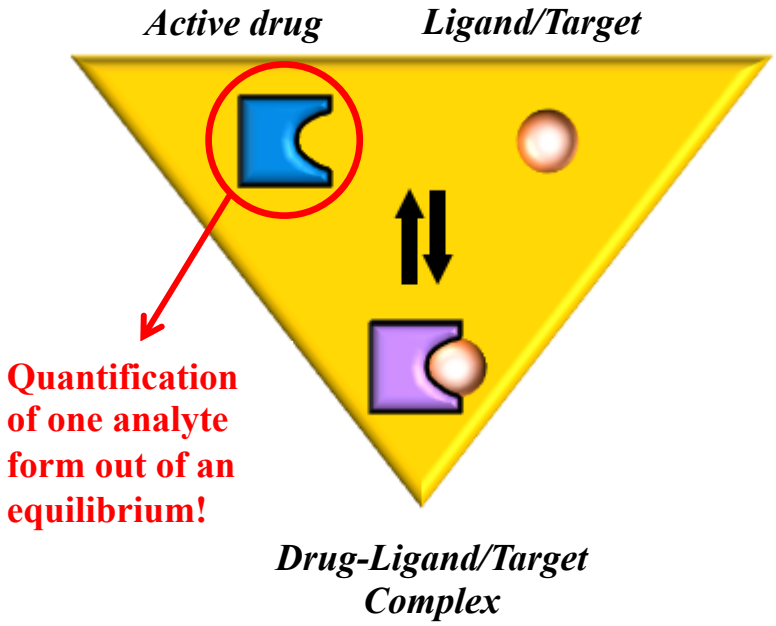
Active Drug Quantification

Why Active Drug Quantification Matters ...

In vivo situation for drug in matrix







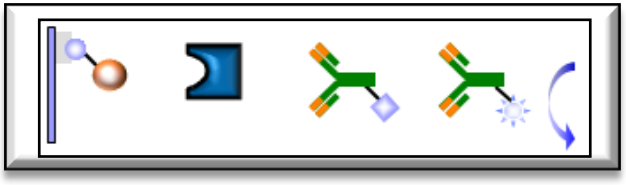
<u>Active drug:</u> Relevant species for pharmacokineticists for a thourough understanding of the **PK/PD relationship**

<u>Total drug:</u> Can be relevant, especially in pre-clinical stage, because **toxicological effects** may pose safety concerns

Active Drug Quantification

Basic Requirements for Accurate Active Drug Quantification

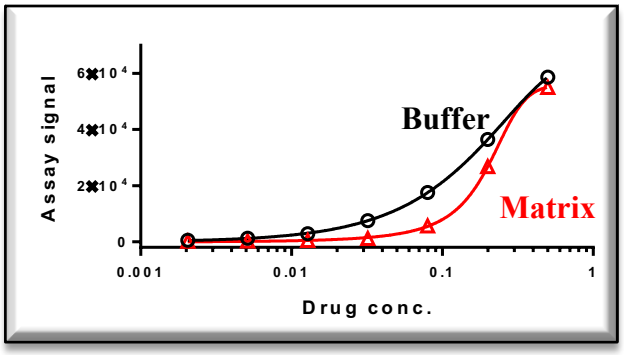
1. Assay Concept



Appropriate assay format and conduct

- Capture/detection reagents
- Assay protocol

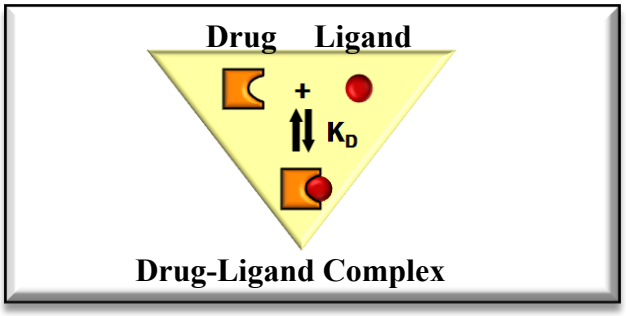
2. Matrix Interference and Calibration Concept



Wanted vs. unwanted matrix interference

- Use of surrogate ligand-free matrix for defined calibration standards and QC samples

*3. Free analyte QC concept**



Accurate and precise active drug quantification

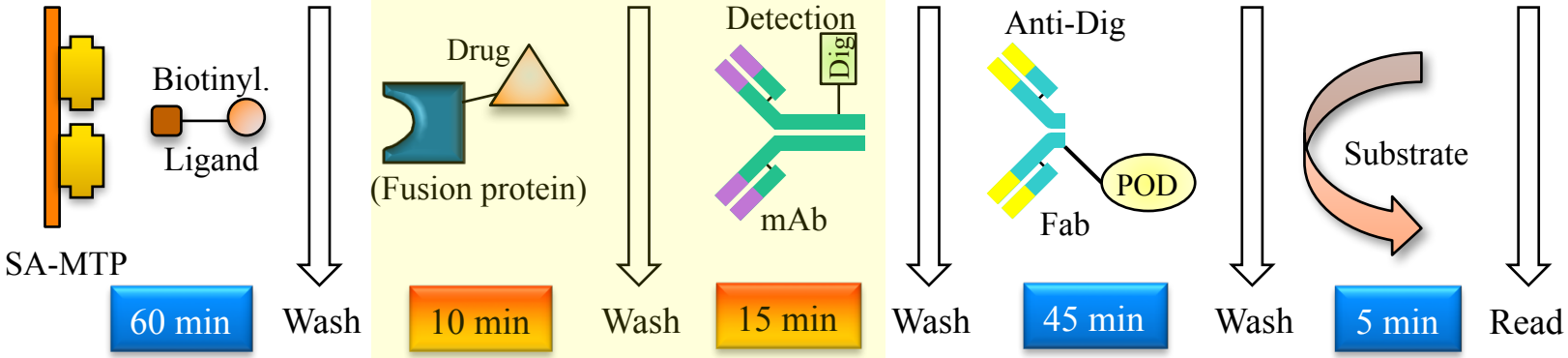
- Preparation of QC samples with exactly defined active drug concentrations (and different drug-ligand ratios)

* *Staack RF, Jordan G, Dahl U, Heinrich* J. Bioanalysis. 2014 Feb; 6(4): 485-96

Active Drug Quantification

Assay Concept

Accurate and precise active drug quantification in monkey plasma



Dedicated Assay Reagent Concept

- Combination of capture and detection reagents bind/detect only active drug with free binding and/or effector domains
- **Special calibration and QC sample concept**

Optimized Assay Protocol

- Rapid sample dilution with immediate subsequent assay conduct
- Short incubation times for diluted samples on MTP and detection mAb

Active Drug Assays and Current Guidelines

Challenges/Limitations

Active drug assays can be validated according to current PK assay validation guidelines (EMA, FDA, …) for some aspects

However, the guidelines do not consider special needs of active drug assays for validation and sample analysis:

- General validation concept
- Analytical run acceptance criteria
- Investigation of matrix effect and conclusions
- Calibration concept
- QC sample concept
- Assessment of dilution linearity and parallelism
- Stability assessment in matrix

Active Drug Assays and Current Guidelines

*Validation Concept for Active Drug Assays**

Species-independent validation approach

- **Validation of intrinsic assay parameters** performed in surrogate matrix (**different species lacking cross reactive ligand**)
  - Sensitivity and calibration range
  - Assay precision and accuracy
  - Dilution linearity, prozone effect
- **Validation of critical species-specific parameters** requiring the 'actual' matrix performed for
  - Selectivity — Ligand-depleted individual matrix
  - Dilution parallelism — Ligand-depleted individual matrix
  - Incurred samples reanalysis (ISR) — Actual study samples

Acceptance criteria for validation parameters according to FDA and EMA guidelines

Challenges for active drug assays:

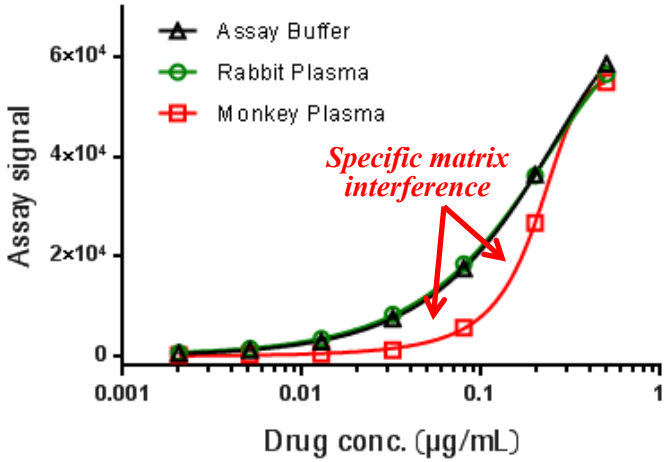
- Validation concept not covered by currently existing guidelines

* *Schick E, Staack RF, Haak et al.* J. Bioanalysis. 2016 Nov; 8(24): 2538 - 2549

Active Drug Quantification

Calibration Concept

Analysis of calibration standards in buffer and naive vs depleted monkey plasma



	Pooled cynomolgus monkey plasma			
	Naive		Ligand-depleted	
Nominal conc. (µg/mL)	Found conc. (µg/mL)	Accuracy (%)	Found conc. (µg/mL)	Accuracy (%)
0	BLQ	N/A	BLQ	N/A
0.00205	0.00277	135.3	0.00175	85.4
0.00512	0.000702	13.7	0.00463	90.4
0.0128	0.00197	15.4	0.0121	94.5
0.0320	0.00641	20.0	0.0310	96.9
0.0800	0.0282	35.3	0.0826	103.3
0.200	0.139	69.5	0.212	106.0
0.500	0.447	89.4	0.517	103.4

Specific matrix interference observed for monkey plasma caused by endogenous ligand

- Actual matrix (naive monkey plasma) thus not suited for calibration

- **Ligand free plasma** required for accurate drug quantification
- **Surrogate plasma** of a different species lacking (cross-reactive) ligand was selected for calibration standard and QC sample preparation

Active Drug Assays and Current Guidelines

Matrix Selection, Calibration Concept and QC Sample Preparation

Current contents

EMA Guideline:

4.1.4. Calibration curve

The response of the instrument with regard to the concentration of analyte should be known, and should be evaluated over a specified concentration range. The calibration standards should be prepared in the same matrix as the matrix of the intended study samples by spiking the blank matrix with known concentrations of the analyte. There should be one calibration curve for each analyte studied in the method validation and for each analytical run.

7.1.1.5. Matrix selection

The measurement of some macromolecules may not be possible in complex matrices without extraction due to high interferences with high levels of structurally related endogenous compounds. Although the use of extracted matrix (e.g. charcoal, immuno-affinity) or alternative matrix (e.g. protein buffers, dialysed serum) is not recommended, the use of such matrices may be necessary when there is no other strategy to quantify the analyte of interest. The calibration standard curve may be prepared in these surrogate matrices. QC samples should be prepared in the actual sample matrix and the accuracy should be calculated to demonstrate the absence of matrix effect.

FDA Guideline:

sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the

concentrations that are below the established LLOQ and above the established ULOQ. Whenever possible, calibrators should be prepared in the same matrix as the study samples or in an alternate matrix of equivalent performance. Both ULOQ and LLOQ should be defined by acceptable accuracy, precision, or confidence interval criteria based on the study requirements.

- The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.

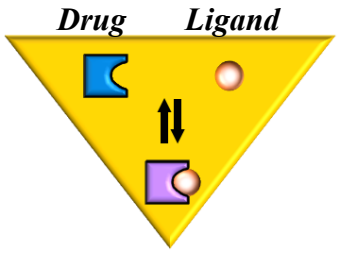
Challenges for active drug assays:

- Investigation and discrimination of matrix interference
  - **Specific** vs **non-specific interference** (wanted vs unwanted matrix effect)
- **Selection of the right matrix** (surrogate matrix) for calibration standard preparation
- Free analyte QC sample preparation not covered by current guidelines

Active Drug Quantification

Free Analyte QC Concept

Preparation of **free analyte QC samples in surrogate matrix** with exactly defined **free drug concentrations**, which are in equilibrium with drug-ligand complexes



Drug-Ligand Complex

Law of Mass Action

$$K_D = \frac{[A_{free}] \cdot [B_{free}]}{[AB]}$$

	Free analyte QC samples (drug + ligand)				
	LLOQ	**LQC**	**MQC**	**HQC**	**ULOQ**
Nominal *free* conc. (µg/mL)	0.00205	0.006	0.200	0.375	0.500
Free drug fraction (%)	**20.5**	**60.0**	**40.0**	**18.8**	**25.0**

+ second set of **total analyte QC samples**
(for comparison purposes)

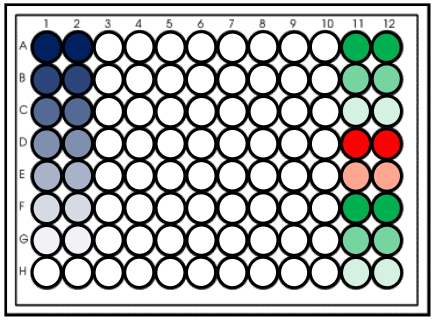
	Total analyte QC samples (drug only)				
	LLOQ	**LQC**	**MQC**	**HQC**	**ULOQ**
Nominal *total* conc. (µg/mL)	0.00205	0.006	0.200	0.375	0.500
Free drug fraction (%)	**100**	**100**	**100**	**100**	**100**

Staack RF, Jordan G, Dahl U, Heinrich J. Bioanalysis 2014 Feb; 6(4):485-96

Active Drug Assays and Current Guidelines

Assay Conduct and Analytial Run Acceptance Criteria

Microplate Layout



- Calibrator
- Total QC samples (drug)
- Free QC samples (drug + ligand)
- Validation/study samples

- 7 calibration standards + blank
- 2 sets of total analyte QC samples (low/mid/high)
- 1 set of free analyte QC samples (low/high)

Analytical Run Acceptance

Calibration standards

- ≥6 concentration levels
- Accuracy 80%-120% (75%-125% at LLOQ)

Total analyte QC samples

- 4-6-20 rule, ≥1 QC per concentration level

Free analyte QC samples

- 80-120% accuracy at both levels

Calibration standard curve: Prepared fresh daily

QC/ validation samples: Frozen at -80°C at least over night

Requirement for active drug assays:

➢ Addition of free analyte QC samples for analytical run acceptance

Active Drug Assays and Current Guidelines

Assay Precision and Accuracy

	Total analyte QC samples					Free analyte QC samples				
	LLOQ	Low	Mid	High	ULOQ	LLOQ	Low	Mid	High	ULOQ
(µg/mL)	0.00205	0.006	0.200	0.375	0.500	0.00205	0.006	0.200	0.375	0.500
Free drug fraction (%)	100	100	100	100	100	20.5	60.0	40.0	18.8	25.0
	Intra-Assay					Intra-Assay				
Mean conc. (µg/mL)	0.00220	0.00583	0.213	0.387	0.515	0.00159	0.00564	0.215	0.399	0.536
Mean accuracy (%)	107.3	97.2	106.5	103.2	103	77.6	94.0	107.5	106.4	107.2
Precision (% CV)	4.7	3.0	5.1	3.2	6.5	7.7	4.6	3.0	2.8	6.2
	Inter-Assay					Inter-Assay				
Mean conc. (µg/mL)	0.00192	0.00530	0.206	0.371	0.510	0.00173	0.00533	0.216	0.383	0.500
Mean accuracy (%)	93.7	88.3	103	98.9	102	84.4	88.8	108	102.1	100
Precision (% CV)	2.7	3.9	6.5	3.0	3.0	4.5	3.7	2.7	2.4	5.7

Requirement for active drug assays:

- Precision and Accuracy Assessment to be performed for
  - **Total analyte QC samples** (general proof of acceptable P&A for total drug)
  - **Free analyte QC samples** (proof of acceptable P&A for **free drug in coexistence with complexed drug)**

Active Drug Assays and Current Guidelines

Selectivity and Specificity

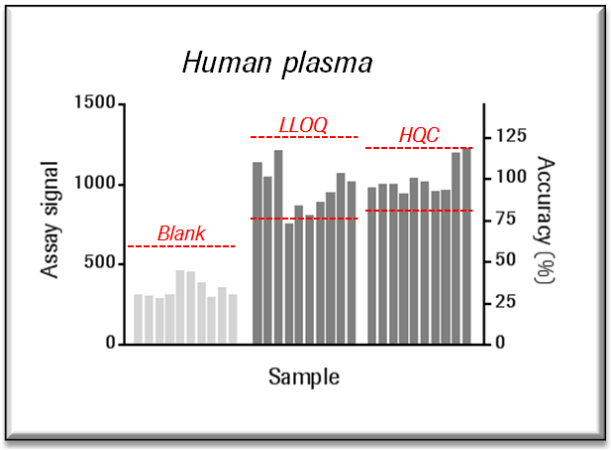
Current contents

EMA Guideline:

antibodies or rheumatoid factor, may interfere with the analyte of interest in the ligand binding assay. Selectivity is tested by spiking at least 10 sources of sample matrix at or near the LLOQ. These sources

FDA Guideline:

presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be



Selectivity assessment to be performed using **ligand-depleted matrix** to mimick the nature of study samples as closely as possible

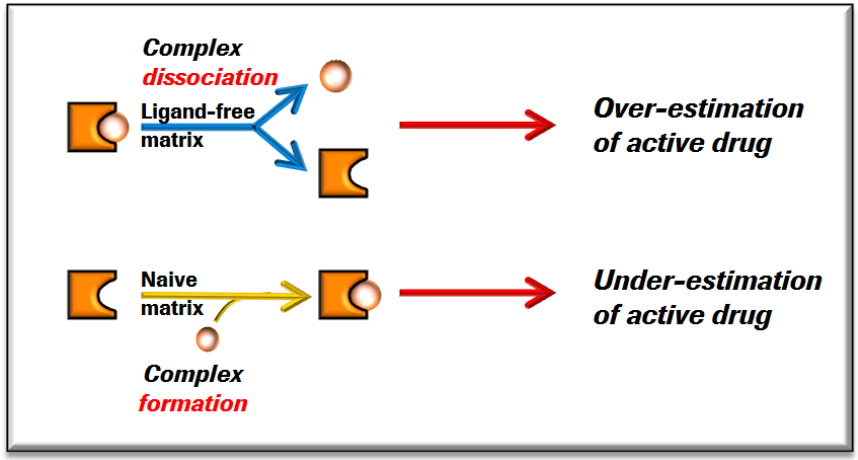
Requirement for active drug assays:

- **Use of depleted matrix** should be considered as basic requirement for selectivity/specificity assessment in active drug assay validation

Active Drug Assays and Current Guidelines

Dilution Linearity

Key parameter for active drug assay validation!



Current contents

EMA Guideline:

Dilution of samples should not affect the accuracy and precision. If applicable, dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria, i.e. within ±15%. Dilution integrity should cover the dilution applied. Evaluation of dilution integrity may be covered by partial validation. Use of another matrix may be acceptable, as long as it has been demonstrated that this does not affect precision and accuracy.

bring the analyte concentrations into the validated range for analysis. An additional reason for conducting dilutional experiments is to detect a possible prozone or "hook effect" i.e. a signal suppression caused by high concentrations of analyte. The back-calculated concentration for each

FDA Guideline:

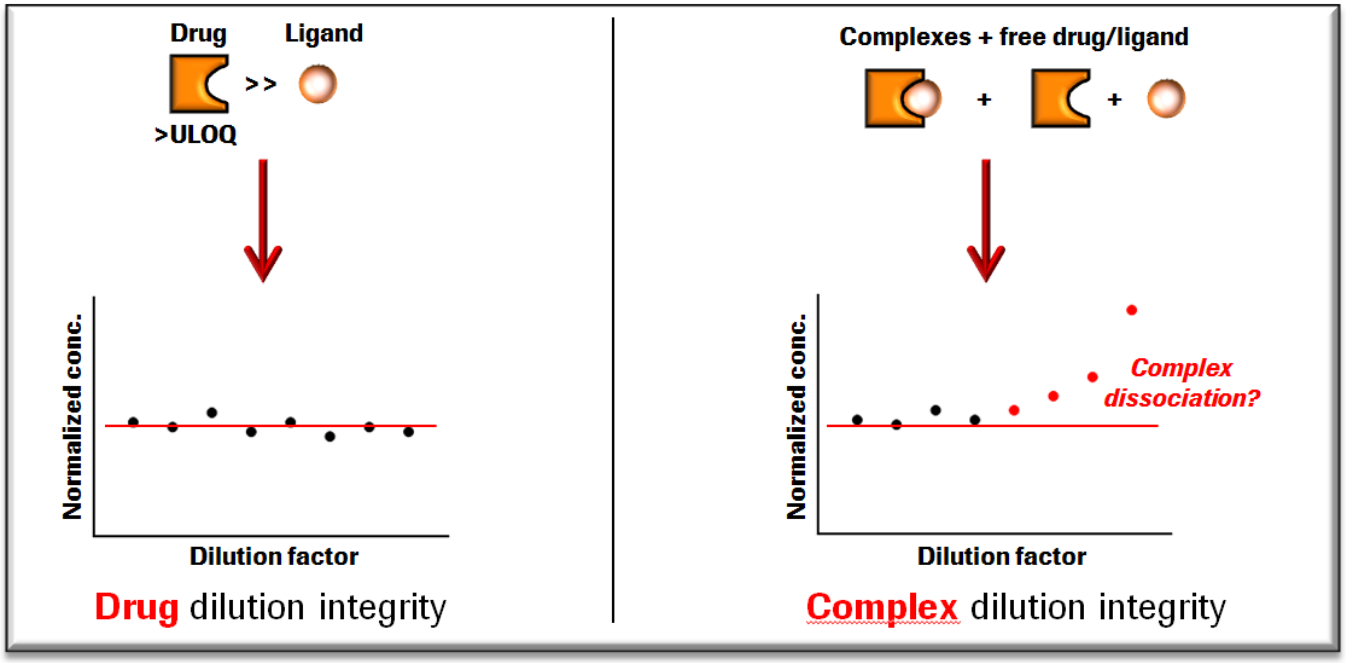
- The dilutional linearity to the reference standard should be assessed using study (incurred) samples.

Influencing factors:

- Type of sample diluent (naive matrix, ligand-free surrogate matrix)
- Sample dilution factor (extremely variable, between 10 and 1'000'000 for ELISAs)
- Time between sample dilution and assay conduct
- Incubation times on microplate

Active Drug Assays and Current Guidelines

Dilution Linearity



Requirement for active drug assays:

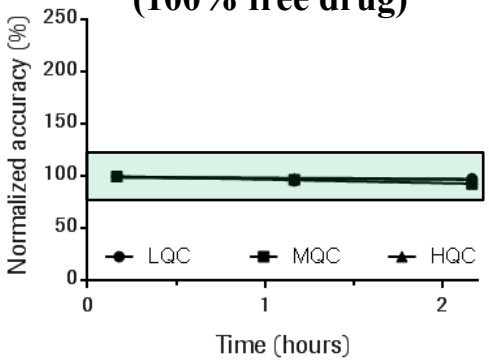
- **Two types of samples required** for dilution linearity assessment
  - To prove dilution linearity of drug in matrix
  - To exclude peturbation of drug/ligand complex equilibrium caused by sample dilution
- **Ligand-free (surrogate) matrix** to be used for sample dilution

Active Drug Assays and Current Guidelines

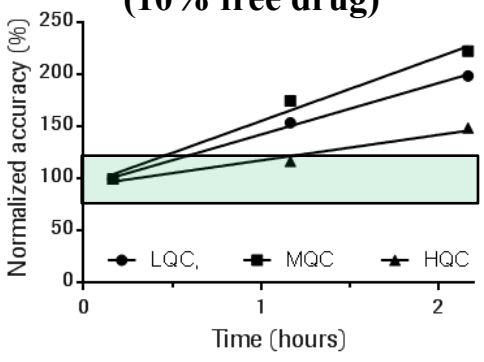
Dilution Linearity – Time Matters …

Time course of drug/ligand or drug/ADA complex dissociation after sample dilution

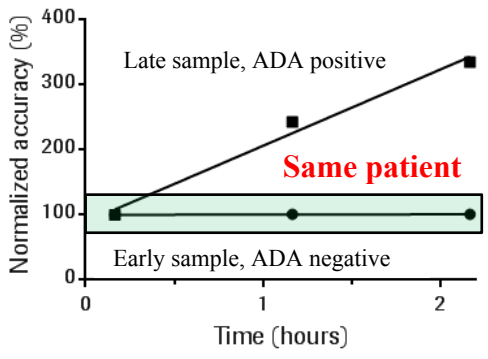
Total analyte QC samples
(100% free drug)



Free analyte QC samples
(10% free drug)



Clinical samples

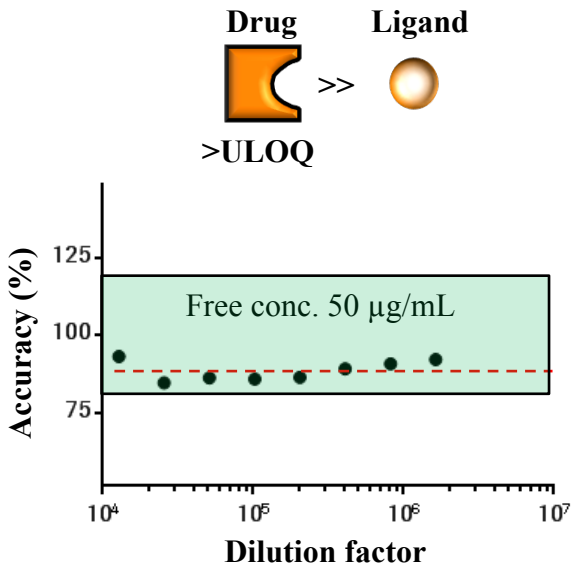


Drug/ligand and drug/ADA complex dissociation after sample dilution depends on

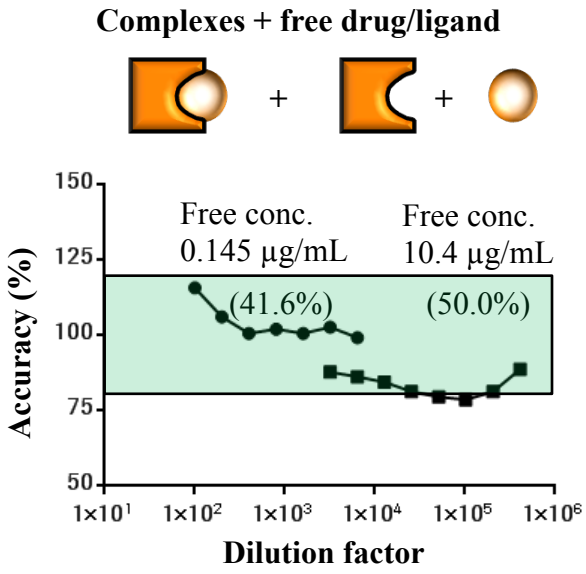
- Dilution factor
- Absolute drug, ligand and ADA concentration
- Fraction of complexed drug
- Temperature
- Dissociation constant K_D

Active Drug Assays and Current Guidelines

Dilution Linearity



Mean accuracy: ***88.6%***
Precision (CV): ***3.7%***



Mean accuracy: ***103.9% / 83.6%***
Precision (CV): ***5.3% / 4.6%***

- Dilution linarity proven up to a dilution factor of 1.6 Mio
- No significant perturbation of drug/ligand complex equilibria observed

Active Drug Assays and Current Guidelines

Dilution Parallelism

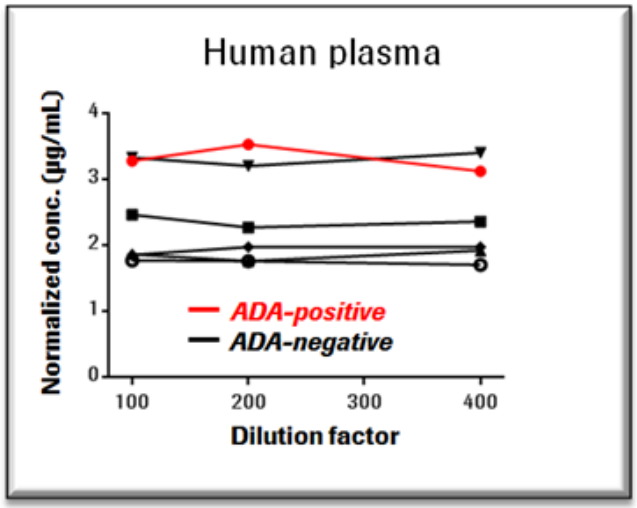
Current contents

EMA Guideline:

study samples should be assessed to detect possible matrix effect or differing affinities for metab
A high concentration study sample (preferably close to Cmax) should be diluted to at least three concentrations with blank matrix. The precision between samples in a dilution series should not e
30%. In case the sample does not dilute linearly (i.e. in a non parallel manner), a procedure for

FDA Guideline:

The dilutional linearity to the reference standard should be assessed using study (incurred) samples.



Requirement for active drug assays:

- **Different types of samples required** for dilution parallelism assessment
  - ADA-negative and positive samples
  - Samples containing drug/ligand complexes with free drug fractions far below 100%
  - Whole study sample concentration range to be covered by dilution parallelism testing
- **Ligand-free (surrogate) matrix** for sample dilution

Active Drug Assays and Current Guidelines

Stability Assessment

- Beside **drug stability** testing, **drug-ligand complex stability** assessment is crucial for active drug assay validation
- Stability of protein drug can be assessed using the total drug assay
- F/T Stability of drug-ligand and drug-ADA complexes was proven using the active drug assay by
  - Free analyte QC sample analysis
  - Dilution linearity testing
  - ISR assessment
- More detailed characterization of drug-ligand complex stability required
    - 3x freeze/thaw stability
    - 24 hours benchtop stability
    - Storage stability at -20°C and/or -80°C

Summary and Conclusions

- A significant fraction of highly potent protein drug can be present in a complexed form, bound to soluble ligand and/or ADA
- Knowledge of the active drug concentration as the relevant species is thus essential for the understanding of the PK/PD relationship
- Validation of an active drug assay was successfully demonstrated by applying the free analyte QC sample concept
  - Ligand-free calibration for accurate quantification
  - Free analyte QC samples containing drug-ligand complexes together with exactly known free drug concentrations
  - In-depth dilution linearity and parallelism assessment to prove accurate free drug quantification over a broad drug concentration range

Summary and Conclusions

- Some validation parameters of active drug assays can be assessed according to the guidelines, but the following special requirements need to be considered:
  - General validation concept
    - Validation of species-independent parameters in surrogate matrix (intrinsic assay parameters)
    - Validation of species-specific parameters in actual (depleted) matrix
  - Assessment of matrix effect (specific vs non-specific interference)
  - Selection and use of (ligand-free) surrogate matrix for calibration standard and QC sample preparation
  - Requirement for free analyte QC samples for analytical run acceptance
  - Dilution linearity/parallelism assessment (also for drug-ligand complexes)
  - Stability assessment of drug-ligand complexes

Acknowledgement

pRED Pharmaceutical Sciences, Large Molecule Bioanalytics at Roche Innovation Centers Basel and Munich

Roland Staack

Gregor Jordan

Uwe Dahl

Markus Haak

Janina Thiele

Julia Heinrich

Herbert Birnboeck

Apollon Papadimitriou

Lisa Benicosa

Thomas Singer

Doing now what patients need next