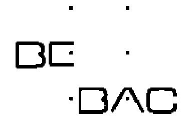


# Bioanalytical Method Development and Validation


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# Method Development: Before one starts…

- Communciation with the clinical site
  - Which concentrations are expected and how long will samples be stored?
- The method should be fit for the intended use – no need to have a 'perfect' method
  - Reliable and reproducible according to the goalposts set in the validation guidelines
  - Intended use in BE
    - LLOQ possible to detect carry-over (≤5% $C_{max}$ in any subject)
      $AUC_{0-t}/AUC_{0-\infty}$ ≥80%
    - ULOQ approx. at the expected $C_{max}$ in any subject
    - A & P

| | | |
|---|---|---|
| chromatography | at the LLOQ | 20% |
| | above | 15% |
| LBAs | at the LLOQ | 30% |
| | above | 20% |

# Which Analyte?

- Parent *vs*. Metabolite
  - Generally parent drug
    - Concerns, that $C_{max}$ of metabolite does not reflect difference in the rate of absorption between formulations
  - If method is not sensitive enough a higher single dose can be considerd
  - Metabolite acceptable 'in exceptional cases'
    - Does not distinguish between active and inactive metabolites
    - Present any available data supporting the view that the metabolite exposure will reflect parent drug
    - Metabolite formation is not saturated at therapeutic doses

# Which Analyte?

- Pro-Drugs
  - High clearance (very fast half-live), very rapidly metabolized
  - If ever possible
    - the inactive pro-drug
    - Active metabolite does not need to be measured
  - If pro-drug has low concentrations and is quickly eliminated
    - Base BE on active metabolite
    - Pro-drug does not need to be measured
  - Parent compound can be considered to be an inactive pro-drug if it has no or very low contribution to clinical efficacy

# Which Analyte?

- Chiral *vs*. Achiral
  - If API in the formulation is a single enantiomer *and* no *in vivo* interconversion documented, an achiral method is sufficient
    - If interconversion or unknown, chiral (enantioselective) method
      - BE based on the active enantiomer
      - Inactive enatiomer as supportive information
  - If the formulation contains a racemate
    - Achiral method generally sufficient
    - 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

# Basics of Bioanalytics

- Biological matrices
  - Whole Blood
  - Serum
  - Plasma
  - Urine
  - Liquor, Saliva, Synovia, Tissue, Faeces, Sputum,…
- Sample Preparation
  (Extraction / Trace Enrichment Techniques)
  - Dilution
  - Protein Precipitation
  - Liquid-Liquid Extraction (LLE)
  - Solid Phase Extraction (SPE)

# Basics of Bioanalytics

- Separation and the ‘Workhorses’ in Bioanalytics
  - for ‘Small Molecules’: Chromatographic Methods
    - High Performance Liquid Chromatography (HPLC/UHPLC)
    - Gas Chromatography (GC)
    - High Performance Thin Layer Chromatography (HPTLC)
    - Capillary Electrophoresis (CE)
    - Supercritical Fluid Chromatography (SFC)
  - Hyphenated Techniques
    - Column Switching (LC/LC, GC/GC, SFC/GC)
    - MS-MS, TOFMS
  - for Biologics: Ligand Binding Assays
    - Enzyme Linked Immunosorbent Assay (ELISA),
      Enzyme Multiplied Immunoassay Technique (EMIT)
    - Radioimmunoassay (RIA)

# Basics of Bioanalytics

- Detection
  - for HPLC
    - Ultraviolet / Visible (UV/Vis), Diode-Array (DAD)
    - Fluorescence (FL)
    - Electrochemical (EC: coloumetric and amperometric)
    - Mass Spectrometry (MS)
    - Conductivity, Diffraction (Evaporative Light Scattering, ELS), Refractive Index (RI), Radioactivity (RA), …
  - for GC
    - Flame Ionization Detection (FID)
    - Nitrogen-Phosporus Detector (NPD)
    - Electron Capture Dector (ECD)
    - Mass Spectrometry (MS)
    - Thermal Conductivity (TCD)

# Basics of Bioanalytics

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

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

    Ideally $MF \sim 1$
  - Protein-binding
    - It's an urban myth that – at least for BE studies – free (unbound) drug should be measured, even for highly protein bound drugs
    - Common sample preparation techniques lead *always* to the total concentration

# Biological Matrices

- Whole Blood
  - Should be avoided if ever possible …
  - Mandatory if drug binds strongly to erythrocytes, *i.e.*, plasma concentrations cannot be measured (example chlorthalidone)
  - Recommended if drug is routinely measured in Therapeutic Drug Monitoring (TDM) in whole blood, even if measurement in plasma is possible.
    Examples: tacrolimus; ciclosporin, bortezomib?

# Biological Matrices

- Serum
  - Only if no stability issues
    (sufficient time for clotting – cooling not allowed)
  - Possible problems after thawing (turbidity requiring centrifugation – rarely labs are equipped with a cooled centrifuge)
  - Sometimes problems become evident only in multiple freeze-thaw-cycles (part of validation, but not of method development)

# Biological Matrices

- Plasma
  - Better choice than serum, because whole blood can be cooled immediately after drawing until centrifugation.
  - Numerous anticoagulants available
    - EDTA salts (Na, K, Li)
    - citrate
    - heparin
  - Anticoagulant must no interfere with the extraction / separation / detection!

# Biological Matrices

- It is the job of the bioanalyst to
  - perform pre-tests of stability (from sampling until thawing)
    - Important is stability from blood draw → centrifugation → freezing
    - Recommended procedure
      - Spike whole blood at 37 °C with the analyte(s)
      - Centrifuge immediately → analyze plasma (best case)
      - Keep other samples for increasing time intervals → centrifuge → analyze
      - If the first sampe show substantial degradation, a stabilizer has to be found
      - If analyte is stable for a certain interval (and degrades later) → limit for the sampling procedure
      - If degradation sets in to early, try puting whole blood on ice
  - find suitable storage conditions (–20 °C, –80 °C)
  - Conditions have to be cleary communicated with the clinical site

# Biological Matrices

- Urine
  - Quite often clinical sites fill urine sample vials up to the stopper and forget the 9% increase in volume (water → ice) …
  - All analytes must be dissolved after thawing
    - Hints
      - Increase the temperature to 37 °C (if stability allows) and opt for a shaker or ultrasonicifaction
      - Dilution with $H_20$ helps

# Sample Preparation (Extraction/Trace Enrichment)

- Dilution
  - For urine samples – with a few exceptions:
  - Example: triamterene in plasma
    - Solubility 48 mg/mL
    - Hydrophobicity logP
      0.3 (measured), 1.21 (predicted): extremely hydrophilic!
      - LLE not possible
      - SPE almost not possible as well
    - Acidity pKa 6.2
    - Protein binding 97%
    - 200 µL plasma diluted with 600 µl $H_20$ → 20 µl HPLC
      NH2 column, fluorescence 360 nm excitation / 436 nm emission
      LLOQ 1 ng/mL

# Sample Preparation (Extraction/Trace Enrichment)

- Protein Precipitation
  - A.k.a. 'point-and-shoot'
    - First option in LC/MS-MS if a stable-isotope internal standard is available
    - As a last resort in LC/MS-MS with structural analogue internal standards (only if MF ~1)
  - Rough treatments (perchloric acid, trichoroacetic acid) should be avoided
    - Analyte will be trapped in denaturized protein clots
  - Suitable (especially for LC/MS)
    - Acetonitrile (recommended), methanol, or ethanol
  - All other low molecular mass compounds (incl. endogenous ones) remain in solution
    - Only the best choice for good separation and selective detection

# Sample Preparation (Extraction/Trace Enrichment)

- Liquid-Liquid Extraction (LLE)
  - Based on distribution between an aqueous phase (plasma, urine) and an organic phase
  - Driven by lipophilicity of the analyte
    - Octanol-water partition coefficients are part of pharmacopoeial information, but only useful for neutral molecules
  - Given as the decadic logarithm *log* P; the higher, the more lipophilic
    - Examples

| Drug | *log P* |
|---:|---:|
| Caffeine | –0.23 |
| Paracetamol (Acetaminophen) | 0.51 |
| Diclofenac | 4.98 |
| Montelukast | 7.26 |

# Sample Preparation (Extraction/Trace Enrichment)

- Liquid-Liquid Extraction (LLE)
  - If the analyte consists of an organic core and at least one acidic/basic group, the pH of the the aqeous phase drives the process
  - Dissociated → dissolved salt → hydrophilic
  - Not dissociated → organic core → lipophilic
  - Amphoterics (containing acidic and basic group) are problematic
    - Multiple steps at different pHs may be necessary
    - Sometimes it's not possible to find a pH which gives sufficient recovery
  - However, a recovery close to 100% is not required
    - If the method is sensitive, accurate, and precise enough – *any* recovery is acceptable

# Sample Preparation (Extraction/Trace Enrichment)

- Solid Phase Extraction (SPE)
  - Based on distribution between an aqueous phase (plasma, urine) and chemically bonded solid phase matrix
  - Most common reversed phase silica
    - C18, C8, C4, C2, Phenyl
  - Rarely normal phase
    - NH2, OH, CN
  - Selective washing steps possible
  - Can be automated – or even linked to the main HPLC-system (column switching)

# Chromatography

- GC *vs*. HPLC
  - GC
    - \+ For volatile and temperature-insensitive compounds (example valproic acid)
    - \+ Easy coupling to MS (analyte already in gas phase)
    - \+ High separation power (capillary length up to 50 m)
    - \+ Fast analyses possible (with short columns only restricted by the detector and data system)
    - \+ Negligible matrix effects in MS (small absolute amounts injected and high selectivity)
    - – Non volatiles require derivatization (majority of drugs; expertise needed)
    - – Chiral columns expensive and delicate

# Chromatography

- GC *vs*. HPLC
  - HPLC
    - \+ Method of choice for non-volatile and/or temperature-sensitive compounds
    - \+ Most common method nowadays
    - ± Fast analysis times (UHPLC, particle size <3 µm)
    - ± Coupling to MS (800× more volume than in GC)
    - ± Matrix effects in MS (may require sophisticiated sample preparation)
    - – Highly hydrophilic basic/acidic compounds problematic (ion exchange instead of RP; needs expertise)
    - – Highly hydrophilic neutral compounds require derivatization
    - – Chiral columns expensive and delicate

# HPLC

- Recommendations
  - Resolution between two adjacent peaks >2
  - Tailing factor <2 (higher may be acceptable for chiral methods, where columns show 'bad separation' in general)
  - Run times
    - The longer, the better the separation – but peak heights will decrease (band broadening → worse LLOQ)
  - Run times are *decreased* by
    - Type of stationary phase C18 → C8 → C2
    - Column length ↓
    - Particle size 3 µm → 5 µm
    - Flow rate ↑
    - Type of organic modifier in mobile phase $CH_3OH$ → $CH_3CN$
    - Percent of organic modifier in mobile phase ↑
    - Temperature ↑

# LC/MS-MS (Matrix Effects)

- Minimization
  - Use a stable isotope–labeled internal standard
    - $^{2}H$, $^{15}N$, $^{18}0$ at 3 – 6 positions: different *m*/*z* but similar extraction and chromatography
  - Othewise an internal standard of similar structure
    - Neutral radicals (*e.g.*, $-CH_3$, $-C_2H_5$) preferred
    - Radicals of different polarity/pK less suitable (*e.g.*, -OH, $NH_2$),
    - In this case avoid ‘precipitate and shot’–methods
  - Conduct sufficient sample cleanup – especially to remove phospholipids
  - Use weakly acididic wash solution for on-line SPE negative ion methods to break up Na+/analyte ion pairs
  - Use new chromatographic methods to enhance separation (UPLC, Rapid Resolution LC)

# LC/MS-MS (Matrix Effects)

- Examination
  - Extract different sources 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 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
  - Matrix Factor
    ~1 no matrix effects<br>
    <1 ion suppression<br>
    >1 ion enhancement *or* analyte loss<br>
    in the presence of matrix during analysis

# LC/MS-MS (Other Problems)

- Metabolites
  - Some metabolites dissociate in the ion-source resulting in the parent drug's m/z ion.
  - If these metabolites are not separated from the parent drug in chromatography, one can not distinguish between parent and metabolite in MS.
    - Examples of documented back-conversion: acyl-glucuronides, esters, N-oxides, lactone-rings
  - Since metabolites are often not commerically available, short run-times should be avoided for such drugs
    - Metabolites may be extracted from urine…

# Integration of Peaks in Chromatography

- Peak ‘Recognition’
  - Peak start and end ‘recognized’ by upward-/downward slope detection
    - The data system fits a couple of data points to a function (polynomial, smoothing spline, Savitzky-Golay, …)
    - Calculates the first derivative at each time point
    - If the derivative is positive and above the threshold = start of peak; if the slope is negative and below the threshold = end of peak
    - For a Gaussian peak upward- / downward thresholds would be the same, but in chromatography peaks are never symmetrical
    - Some data systems correct for that by using more slices if the slope is negative or even change to a different fitting algorithm

# Integration of Peaks in Chromatography

- Automatic *vs*. manual
  - Integration parameters are saved in the CDS's method and work in the background
  - The automatic integration may fail
    - Mainly for small peaks close to the LLOQ
    - Rarely for high peaks as well, when a series of positive random noise may trigger an 'end of peak' too early or negative random noise draws the baseline too late
    - There is no 'correct' integration for any given peak! The same raw data most likely will result in different values if exported to another CDS
  - Good practive
    - All chromatograms should be reviewed and the integration adjusted if necessary

# Integration of Peaks in Chromatography

- Automatic *vs*. manual
  - The review should (!) to be performed before concentrations are calculated
    - Changing integration of a peak in order to bring a calibrator / QC sample to a desired value (*e.g.*, force a batch to be valid which would be rejected otherwise) would be clear evidence of fraud
  - Acceptable by current guidelines (FDA 2001, EMA 2011)
    - SOP in place
    - Report which chromatograms were reintegrated (why, by whom, when: all the usual points needed for an audit trail)

# Integration of Peaks in Chromatography

- Automatic *vs*. Manual
  - Example LC/MS-MS
    - Risperidone
    - Protein precipitation
    - Dilution factor 8
    - API 4000, software Analyst 1.4.1
    - 1 ng/mL and 0.1 ng/mL (LLOQ) *

| integration method | 1 ng/mL | 0.1 ng/mL |
|---|---|---|
| | *CV* (*n* = 10) | |
| automated (smoothing 1, bunching 2) | 6.5% | 15.1% |
| manual correction (one analyst) | 6.3% | 11.1% |
| manual correction (ten analysts) | 5.2%<br>(3.8% – 6.8%) | 12.8%<br>(6.9% – 16.0%) |

* Kirchherr H. *Data Evaluation in LC-MS*. In: Kuss H-J, Kromidas S, editors. *Quantification in LC and GC*. Wiley 2009; p243–59.

# Calibration Curve

- Should cover the expected concentration range
  - Most simple function established on re-calculated concentrations
    - Linear, quadratic (LBAs: 4- or 5-parameter logistic)
    - Weighting scheme (common: 1/x, $1/x^2$, $1/y$, $1/y^2$) [1]
    - Assess not only back-calculated concentrations (accuracy and precision) but also the model residuals [1,2]

1 NIST/SEMATECH. *e-Handbook of Statistical Methods*. 4.4.5.2. Accounting for Non-Constant Variation Across the Data. 2012.

2 Almeida AM, Castel-Branco MM, Falcão AC. *Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods*. J Chromatogr B. 2002;774:215–22. doi:10.1016/S1570-0232(02)00244-1.

# Bioanalytical Method Validation (BMV)

- Guidelines
  - FDA (2018, Rev.1 2013, 2001)
  - EMA (2011, 1996)
  - ICH M10 (Draft 2019)
  - WHO (Good Chromatography Practices 2019)
- Two Parts
  - Method Validation
  - Analysis of Study Samples (including ‘Incurred Sample Reanalysis – ISR)

# Bioanalytical Method Validation (BMV)

- Full Validation
  - Selectivity
  - Carry-over
  - Limit of Quantification
  - Calibration
  - Accuracy (A)
  - Precision (P)
  - Dilution Integrity
  - Matrix Effect
  - Stability
  - Recovery (FDA only)
- Partial Validation, Cross Validation
- Validation Report

# Bioanalytical Method Validation (BMV)

- Method Validation Plan
  - Method Development has to be completed
    - Already described in detail or better, an SOP
  - All intended procedures described
    - (Source of) matrix, source of reference standards and IS, extent and duration of stability testing, calibration curve (range, number of calibrators, weighting scheme), QC samples, number of replicates, number of batches, batch size…
    - Limits of acceptance defined
  - Approved by the head of bioanalytics and released by the QAU
    - It is not acceptable to modify – even to improve – the method during validation
    - If that's necessary, start over

# Bioanalytical Method Validation (BMV)

- Selectivity
  - Separation of analyte(s) and internal standard from interferring matrix components
  - At least six different sources of blank matrix
  - Acceptable
    <5% of the LLOQ for the analyte(s)
    <20% for the IS
  - Important: Metabolites and in MS back-converted metabolites (acidic metabolites, unstable N-oxides or glucuronides, lactones)
  - Usally not an issue in BE: Interferences from co-medications

# Bioanalytical Method Validation (BMV)

- Carry-over
  - Even state of the art autosamplers have a carry-over of ~0.5%
  - Inject blank sample after a highest calibrator
  - Acceptable

    EMA

    <5% of the LLOQ for the analyte(s)

    <20% for the IS

    FDA

    <20% of the LLOQ for the analyte(s)
  - If higher
    - additional flush cycles or
    - a blank injection between samples
  - Recommendation: Even if acceptable carry-over, never inject samples in a random order

# Bioanalytical Method Validation (BMV)

- LLOQ
  - Lowest calibration standard which can be measured accurately and precisely
  - Signal should be ≥5times of the blank
  - Six independent sources of matrix
  - At least duplicates
  - Acceptable (back-calculated concentrations)
    - Accuracy * ±20%
    - Precision * 20%
  - Repeated in at least three batches to assess batch-to-batch variability

* Sloppy terminology; actually
Inaccuracy ±20% = Accuracy 80 – 120%
Imprecision 20%

# Bioanalytical Method Validation (BMV)

- Calibration Curve
  - Covers the expected concentration range
  - At least six concentration levels (LBAs: + low/high anchor points) and a blank sample (extracted with and without IS)
  - At least duplicates
  - At least three batches
  - Acceptable
    - Accuracy ±20% of nominal concentration at LLOQ, ±15% above
    - At least 75% of calibrators must pass
    - All back-calculated concentration have to be *reported*
    - In case of replicates not more than 50% at a given concentration level can be excluded
      - If all LLOQ or ULOQ not acceptable → batch rejected
        If next batch fails as well → stop and revise method

# Bioanalytical Method Validation (BMV)

- Calibration Curve (cont'd)
  - Example: Six levels in duplicate, accuracy (% of nominal)
    - 66%|85% (LLOQ),
      94%|88%, 113%|117%, 108%|109%, 102%|94%,
      80%|111% (ULLOQ)
      - mean accuracy: 76%, 91%, 115%, 109%, 98%, 95%
      - 3/12 (25%) can be excluded
    - after exclusion (new regression)
      81% (LLOQ),
      93%|87%, 113%, 109|111%, 103|94%,
      112% (ULOQ)
      - all levels pass the criteria
      - reported mean accuracy: 81%, 90%, 113%, 110%, 99%, 112%

# Bioanalytical Method Validation (BMV)

- Accuracy
  - Blank matrix spiked at four levels (QC samples)
    - Stock solution for spiking independently prepared from the one used in calibration
    - Levels
      - LLOQ
      - low QC (≤3times LLOQ)
      - medium (30 – 50% of the ULOQ)
      - high (≥75 of the ULOQ)
  - Within-run accuracy
    - Single run
      - At least five replicates at each of the four levels
    - Acceptable
      - ±20% of nominal concentration at the LLOQ
      - ±15% all others

# Bioanalytical Method Validation (BMV)

- Accuracy (cont'd)
  - Between-run accuracy
    - At least three runs
      - At least triplicates at each of the four levels
      - One run should have the size of a batch expected in the study (EMA)
    - Acceptable
      - ±20% of nominal concentration at the LLOQ
      - ±15% all others

# Bioanalytical Method Validation (BMV)

- Precision
  - Based on results of QCs used for determining accuracy
  - Expressed as coefficient of variation (*CV*%)
    - Acceptable
      - 20% of nominal concentration at the LLOQ
      - 15% all others

# Bioanalytical Method Validation (BMV)

- Dilution integrity
  - Spike blank matrix above the ULOQ
  - Dilute with blank matrix
    - Acceptable
      - Accuracy ±15%
      - Precision 15%
- Alternative for SPE methods
  (not mentioned in guidelines but widely accepted)
  - Spike blank matrix above the ULOQ
  - Analyze a lower volume
    - Acceptable
      - Accuracy ±15%
      - Precision 15%

# Bioanalytical Method Validation (BMV)

- Matrix effect
  - At least six different sources of matrix (LBAs: more recommended)
    - Spiked at levels of low and high QCs
  - Calculate Matrix Factor (MF) for analyte(s) and IS
  - Alternative for stable isotope labeled IS
    - IS normalized MF = MF of analyte(s) / MF of IS
  - Acceptable
    - CV of MF ≤15%
  - Recommended
    - Additionally
      - one source of hemolized plasma
      - one source of hyperlipidemic plasma

# Bioanalytical Method Validation (BMV)

- Stability
  - Stability of analyte(s) from blood sampling to freezing
  - Stock solution stability of analyte(s) and IS
  - Spiked matrix (level of low QS and close to the ULOQ)
    - Freeze / thaw stability
      (three cycles: freezer ≥12 hours → ambient temperature)
    - Short term stability at ambient temperature
    - Long term stability in the freezer
  - Dried extract or injection phase at storage conditions
  - Autosampler stability

# Bioanalytical Method Validation (BMV)

- Partial valididation
  - In case of minor changes to a validated method only relevant parts have to be validated
  - Examples
    - Higher temperature of the freezer (–70 °C → –20 °C)
      - Freeze / thaw stability
      - Long term stability
    - Change in chromatographic equipment
      - Everything except
        - » Stock solution stability
        - » Stability in matrix
        - » Stability of extracts
        - » Autosample stability
        - » Dilution integrity

# Bioanalytical Method Validation (BMV)

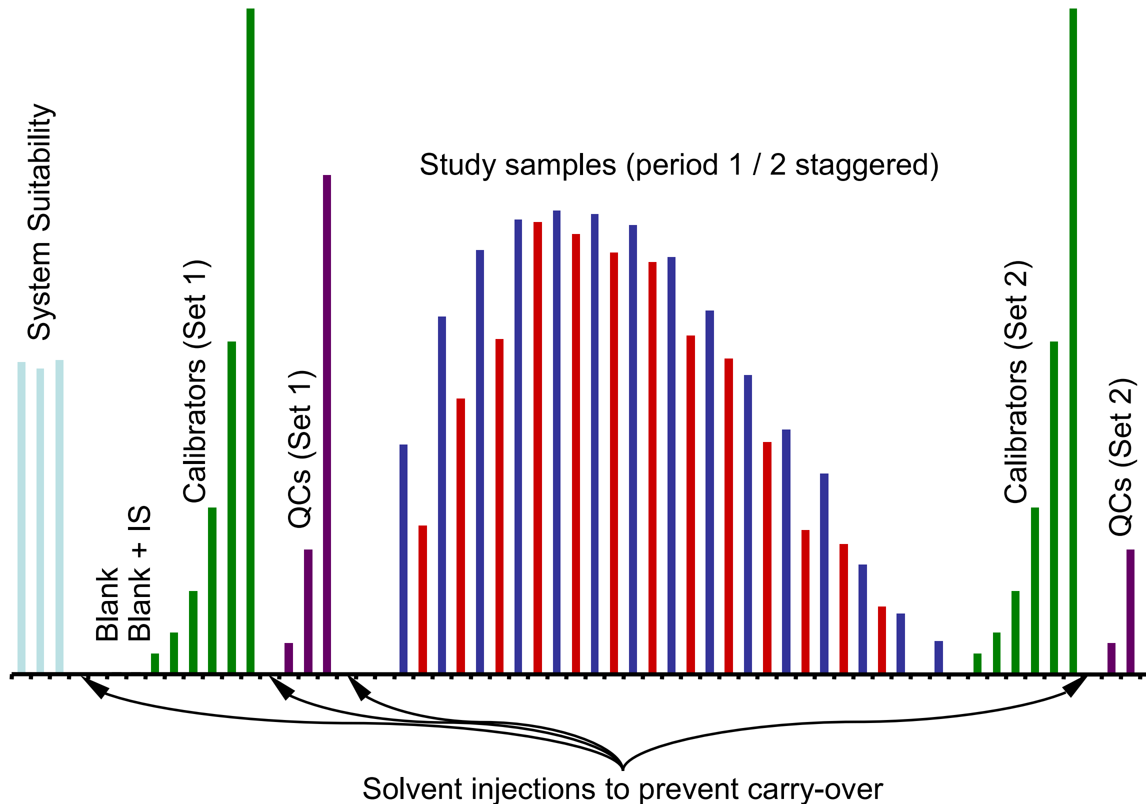
- Analysis of Study Samples
  - Analytical Run (Batch)
  - Acceptance Criteria
  - Calibration Range
  - Reanalysis of Samples
  - Integration
  - Incurred Samples Reanalysis (ISR)
  - Analytical Report

# Bioanalytical Method Validation (BMV)

- Analytical Run (Batch)
  - Definition
    - Samples which are subsequently processed without interruption in time by the same analyst with the same reagents under homogeneous conditions
  - Consists of
    - Blank sample (blank matrix without analyte(s) and IS)
    - Zero sample (blank matrix with IS)
    - Calibration samples (at least six levels)
    - QC samples (low, medium, high; at least duplicates)
    - Study samples
    - Recommended for BE studies
      - All samples of one subject in one analytical run
      - QC samples divided over the run in such a way that A & P of the whole run is ensured

# Bioanalytical Method Validation (BMV)

- Analytical Run (Batch)
  - Example



# Bioanalytical Method Validation (BMV)

- Acceptance Criteria
  - Defined in the analytical protocol
  - Calibration samples
    - At least 75% of calibrators must pass ±15% of the nominal concentration (±20% at the LLOQ)
    - If calibrator(s) rejected, regression re-calculated
  - QC samples
    - At least 67% must pass ±15% of the nominal concentration (±20% at the LLOQ)
    - At least 50% must pass the criteria at each level

# Bioanalytical Method Validation (BMV)

- Calibration Range
  - If a narrow range of analysis values is unanticipated, but observed after the start of sample analysis
    - Analysis stopped
    - Calibration / QCs optimized
    - Analysis continued
    - Not necessary to reanalyze samples bevor the optimization

# Bioanalytical Method Validation (BMV)

- Reanalysis of Samples
  - Possible reasons defined in the protocol or SOP
  - Examples
    - Batch rejected (acceptance criteria for calibrators/QCs not met)
    - IS response in study sampe significantly different from calibrators/QCs
    - Improper sample injection, malfunctioning equipment
    - Sample concentration above ULLQ
    - Quantifiable concentrations in pre-dose samples
    - Poor chromatography
  - Not acceptable in BE studies
    - Pharmacokinetic reasons (irregular profile)
    - However, reanalysis can be performed as part of investigations in order to prevent possible reasons in the future

# Bioanalytical Method Validation (BMV)

- Integration
  - Peak integration and re-integration described in an SOP
  - Any deviation reported and discussed in the report
    - In case of re-integration
      - original data and
      - final data documented

# Bioanalytical Method Validation (BMV)

- Incurred Samples Reanalysis (ISR)
  - Calibrators and QCs do not mimic actual study samples
  - Differences may affect A & P during sample storage, handling, and analysis due to
    - sample inhomogeneity
    - back-conversion of known and unknown metabolites
    - concomitant medications
    - differences in protein-binding
  - A certain fraction of study samples should be reanalyzed
    - If ≤1,000 samples: 10%
    - If >1,000 samples: 100 + 5% of samples exceeding 1,000
    - Two concentration levels
      - around the expected $C_{max}$
      - in the elimination phase

# Bioanalytical Method Validation (BMV)

- ISR (cont'd)
  - Calculate

$$\%\text{difference} = 100 \frac{\text{repeated value} - \text{initial value}}{\text{mean value}}$$

  - %difference should not be greater than 20% for at least 67% of the repeats
  - Larger differences should not lead to rejection of the study by the agency but should be investigated