U.S. FOOD & DRUG ADMINISTRATION

Bioanalytical Method Validation of ANDAs- What the Assessor Looks For

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Outline

- Bioanalytical Method Validation Guidance for Industry (May 2018)
- Method Validation
  - Assessment examples/case studies
- Sample Analysis
  - Assessment examples/case studies
- Conclusions

Method Validation

Specificity

- Blank biological matrix from multiple sources should be analyzed for interference at retention time of analyte (at least 6 sources).
- Matrix effects should be determined (ion suppression or ion enhancement).
- Potential interfering substances in biological matrix should be studied (endogenous matrix components, metabolites, concomitant medications, etc.).

What Does the Assessor Look For?

- Chromatograms have no interfering peaks at retention time of the analyte/internal standard (IS).
- Concomitant medications do not interfere with analyte/IS peak.

Calibration Curve (CC)

- The quantitation range of the assay should be the concentration range expected in the in vivo BE studies
- CC should be continuous and reproducible
- CC should be prepared in the same matrix as the samples in the in vivo BE study

Quality Control Samples (QCs)

- QCs should be prepared in the same matrix as BE study samples
- Recommended QC concentrations are:
  - Lower Limit of Quantification (LLOQ)
  - Low QC (LQC): Three times the LLOQ
  - Mid QC (MQC): Mid-range of CC
  - High QC (HQC): High-range of CC
- CCs and QCs should be prepared from separate stock solutions

Recovery (Extraction Efficiency)

- Recovery of analyte and internal standard (IS) need to be calculated separately. Recovery of the analyte should be calculated at 3 QC levels.
- Recovery should be calculated by comparing the analytical results of extracted samples with corresponding extracts of blanks spiked with the analyte post-extraction, which represents 100% recovery.
- Recovery need not be 100%, but should be efficient and reproducible.
- The Guidance has no listed acceptance criteria for percent recovery. Sponsors should have SOPs in place with their own acceptance criteria and acceptance criteria should be justified.

What Does the Assessor Look For?

- Are percent recovery values consistent across LQC, MQC, and HQC?

Assessment Example

Sample level	replicate	drug response extracted	reference	Recovery (%)
A	1	5830	26003	29.0
	2	4556	23948	22.7
	3	4064	21669	20.2
	4	3197	17733	15.9
	5	2690	15916	13.4
	6	3043	15204	15.2
	Mean		20078.8	19.40
	S.D.		4454.01	5.824
	CV%		22.2	30.0
B	1	902527	4029999	28.9
	2	774307	3703292	24.8
	3	738927	3342169	23.7
	4	590722	2810824	18.9
	5	515835	2532003	16.5
	6	450862	2304322	14.4
	Mean		3120434.8	21.20
	S.D.		681813.25	5.516
	CV%		21.8	26.0
C	1	1246976	5617510	29.4
	2	1214205	4979763	28.6
	3	1171085	4523066	27.6
	4	832666	3662460	19.6
	5	767793	3424851	18.1
	6	729496	3228689	17.2
	Mean		4239389.8	23.42
	S.D.		953485.97	5.686
	CV%		22.5	24.3
	Grand Mean			21.34
	S.D.			5.594
	CV%			26.2

Assessment Example

Sample level	replicate	internal standard response extracted	reference	Recovery (%)
A	1	245426	1280140	26.1
	2	196034	1153023	20.9
	3	178602	1020698	19.0
	4	138425	827002	14.7
	5	119377	748210	12.7
	6	117975	704253	12.6
B	1	215970	1240389	23.0
	2	204798	1112647	21.8
	3	182807	1014564	19.4
	4	130904	844715	13.9
	5	127885	737823	13.6
	6	111384	686874	11.9
C	1	234449	1253164	24.9
	2	223722	1076364	23.8
	3	202144	995904	21.5
	4	148875	792637	15.8
	5	136864	740226	14.6
	6	127864	689365	13.6
	Mean		939888.8	17.99
	S.D.		210961.10	4.772
	CV%		22.4	26.5

Accuracy and Precision (A&P)

- A&P experiments should include a minimum of 3 independent runs conducted over several days.
- Freshly prepared calibrators and QCs should be used for all A&P runs.
- A&P should be evaluated at the LLOQ, LQC, MQC, and HQC.

Stability

- Stability of the analyte should be determined in biological matrix for intended duration of the sample collection, handling, and storage.
- Autosampler, benchtop, processed, freeze-thaw, stock solution, and long-term stability of the analyte should be determined.
- For combination drug products, the stability of the analyte should be assessed in the presence of the other drug(s).
- Stability studies should cover the expected sample conditions before receipt at the analytical lab up until the analysis.

What Does the Assessor Look For?

- Is long term storage stability (LTSS) sufficient to cover the study subject sample storage times?
- Is the same anti-coagulant used for LTSS and during subject sample collection?
- Is autosampler stability sufficient to cover any re-injections?
- Is freeze-thaw stability sufficient to cover any re-assayed subject samples?
- Is stability data sufficient to cover any processing deviations?

Dilution Integrity

- If subject samples have concentrations of the analyte above the upper level of quantification (ULOQ), the integrity of the dilution should be validated.
- QC samples above the ULOQ should be diluted with like matrix to bring the samples within the validated quantitation range.
- A&P of dilution QCs should be demonstrated.

What Does the Assessor Look For?

- Does the dilution integrity concentration cover any subject samples that are re-assayed for concentrations above ULOQ?

Partial Validation

- Partial validations evaluate modifications to the already validated bioanalytical method. Some examples include:
  - Bioanalytical method transfers between laboratories
  - Changes in analytical methodology
  - Change in sample processing procedures
  - Changes in instruments and/or software platforms
  - Extensions of the assay range
  - Changes in anticoagulant (but not changes in the counter-ion) in harvesting biological fluids
  - Changes to the matrices

Endogenous Analytes

- Analytes can be naturally occurring in biological matrices (hormones, insulin, etc.) or come from dietary intake (ions, vitamins, Omega 3 fatty acids, etc.).

- The accuracy of the measurement of endogenous analytes poses a challenge when the assay cannot distinguish between the therapeutic agent and the endogenous counterpart.

- Method should be validated in same biological matrix as subject samples that are free from the endogenous analyte (e.g. stripped plasma).

Endogenous Analytes

- CC should be at expected concentrations of subject samples.
- Endogenous levels of the analyte in the biological matrix should be evaluated before QC preparation by replicate analysis.
- The QCs should account for the endogenous concentrations in the biological matrix (additive) and be representative of the expected study sample concentrations.

BE of Endogenous Analytes: Product Specific Guidance (PSG) for Estradiol Tablets

- BE based on 90% CI of baseline-adjusted Estrone (total).
- Sponsors should submit estradiol (unconjugated) and estrone (unconjugated) data as supportive evidence of comparable therapeutic outcome.
- Supportive data should be submitted for said analytes: individual and mean concentrations, individual and mean pharmacokinetic parameters, and geometric means and ratios of means for AUC and Cmax.

What Does the Assessor Look For?

- Were multiple baseline measurements obtained in the time period before the administration of the study drug?
- When there is dietary intake of the compound, was there strict control of the study subjects' diet?
- Was baseline correction applied to each period?

Back-Conversion of Analyte/Metabolite

- Metabolites can be back-converted to analyte, or vice-versa, prior to or during sample analysis.
- During method development, measures should be taken to stop this process to accurately measure the pivotal analyte/metabolite.
  - Sample collection
  - Sample processing/extraction
  - Sample storage
  - Processed sample in autosampler
- Instability of analyte may also cause failure of Incurred Sample Reanalysis (ISR).

Back-Conversion of Analyte/Metabolite

- Appropriate measures should be taken during method development for unstable analytes/metabolites as well as study sample collection, storage, and analysis.

- Lack of these measures has been a common issue among 32% in BE studies in ANDAs surveyed from 2007-2014.*

*Zhang, Zhen, et.al. A Retrospective Study on the Bioanalysis of Unstable Analytes in the Bioequivalence (BE) Studies Submitted in Abbreviated New Drug Applications (ANDAs), AAPS Conference 2015.

What Does the Assessor Look For?

- Were validation <u>and</u> subject samples treated appropriately to prevent back-conversion (i.e. addition of stabilizers)?
- Was back-conversion assessed during the method validation?

Case Study: Clopidogrel Bisulfate

- Clopidogrel undergoes hydrolysis to form clopidogrel carboxylic acid (CCA). The back-conversion from CCA to clopidogrel could occur in the presence of methanol.*

- Considering that the plasma levels of CCA are considerably higher than those of the parent drug, even a minimal back-conversion of the metabolite would lead to a substantial over-estimation of clopidogrel plasma levels and would bias the outcome of a bioequivalence study.

*Development and validation of an HPLC-MS/MS method to determine clopidogrel in human plasma[J]. Acta Pharmaceutica Sinica B, 2016, 6(1): 55-63

In-Study Analysis

by

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Study Sample Analysis-What Assessor Looks For

- Are CC and QCs included in all analytical runs?
- Does QCs cover the expected sample study concentrations?
- Are QCs interspersed with study samples during processing and analysis?
- If study sample concentrations are clustered in a narrow range of the CC, are additional QCs added to cover the sample range?

In-Study Analysis

- Reanalysis of Study Samples
- Re-integration
- Run Rejections/Re-injections of Study Samples
- Incurred Sample Reanalysis (ISR)

Reanalysis of Study Samples

Documentation to be Submitted

- SOPs including reasons for repeats, number of replicates, acceptance criteria, and incidences that trigger investigations
- Raw numerical data from original and re-assayed runs
- Chromatograms of original and re-assayed samples
- Summary table of sample IDs, reasons for re-assay, original and re-assay values and percent differences, reason(s) for reported values

What Assessor Looks For

- Are there any repeats for pharmacokinetic reasons?
- For assay with multiple analytes, did reanalysis of study samples performed only for analyte with an invalid result?
- Did reanalysis of Calibrators and QC samples performed to bias run acceptance?

Frequently Reported Reasons

- Analyte concentration above the upper limit of quantification
- Analyte concentration is below the adjusted LLOQ in an analytical assay run
- Loss of sample during processing/extraction error
- Internal Standard Variation (IS)
- Poor Chromatography

Case Study- ULOQ

- Large number of repeats (195 samples)
  - ULOQ- 500.237 ng/mL
  - Re-assayed concentrations significantly less than ULOQ for 70% of the samples

Sample	Original Values (ng/mL)	Reassay Values (ng/mL)	Final Reported Values (ng/mL)	Percent of the reported value to the ULQ
A	812.577	171.130	171.130	34.2
B	796.201	228.613	228.613	45.7
C	537.632	187.583	187.583	37.5
D	538.502	197.560	197.560	39.5

- Root cause of the inconsistent assay results was not identified
- Study was repeated due to concern of method reproducibility

Internal Standard Variation

- SOPs should include *a priori* IS variability criteria for reanalysis of study samples.
- IS response should be consistent between subject samples, CCs and QCs.
- For isotopic IS, the concentration values should be similar between original and re-assayed samples.
- Root cause of the variability should be investigated when there is apparent trend or several samples are affected.

Case Study - Internal Standard Variation

- Three runs (25% of study samples) were rejected as CCs and QCs failed for High IS/Low IS response
  - Run Rejection Criteria: ≤ 50% or ≥ 175% of the mean of non-zero internal standard areas
  - Deuterated Internal Standard was used
  - No investigation report was submitted

Case Study - Internal Standard Variation

- Variability attributed to deterioration of the IS solution
- Applicant claimed that no runs were rejected when fresh IS solution was used
- Inspection found this claim to be inaccurate
- Study was rejected, since the validity of the data was questionable

Case Study – Poor Chromatography

- Chromatographic interference in 50% of the runs
- Unable to resolve the co-eluted peak completely based on peak areas
- Five analytical runs failed to meet QCs acceptance criteria based on peak height responses

Interference peaks have an impact on the accuracy of the analyte concentrations. Study data were not acceptable.

Re-integration

Documentation to be Submitted

- Chromatograms from 20% of serially selected subjects
- SOP established *a priori* defining the criteria for re-integration
- Reason for the manual reintegration
- Both original and re-integrated chromatograms along with the data

What Assessor Looks For

- Whether there was selective re-integration of chromatograms without acceptable justifications such as:
  - Retention time shift
  - Co-eluting peak/peak splitting
  - Baseline noise

Run Rejections/Re-injections of Study Samples

What Assessor Looks For

- Rejected Runs
  - Did CCs and/or QCs fail acceptance criteria?
  - Other reasons: Was there an instrument malfunction, column leak, poor chromatography?
- Re-injected Runs
  - Was processed stability demonstrated for the entire duration of the re-injected run?

Case Study – Run Rejection

- Rejected for reason of "Poor Chromatography" of calibration standards

Case Study – Run Rejection

- Samples re-analyzed in new run
  - QCs did not meet the acceptance criteria
- Data from original run was reported
  - Sample volume was insufficient for further reanalysis
- Modifying run and chromatographic acceptance criteria during sample analysis is not acceptable

Incurred Sample Reanalysis (ISR)

Incurred Sample Reanalysis (ISR)

- Conduct on at least 10% of the first 1000 study samples and 5% of the remaining samples.
- Sample selection to ensure adequate coverage of entire PK / PD profile of all subjects.
- Samples should be analyzed in a different run from the original analysis.
- Difference in the concentration values between initial and ISR should be within ±20% of the mean of the two values for at least 67% of ISR results.

Incurred Sample Reanalysis (ISR) cont'd

- ISR values should not be used in final pharmacokinetic analysis.
- Reanalysis of the individual samples of the original assay runs should not be based on ISR failures.
- Run rejections should not be based on the ISR results.
- All ISR investigations should be documented and guided by an SOP.

In Summary

- A written description (SOP or protocol) for the bioanalytical method should be established before initiating the validation.
- A validated method should be used for analysis of subject samples.
- Appropriate partial validation studies should be performed and submitted, in case of modification(s) to validated method.
- SOPs (reanalysis, rejections/re-injections, ISR) should be in place prior to the start of study sample analysis.
- Reasons for repeats should be provided along with supporting documentation.
- Investigation should be conducted per SOP and investigation report should be submitted.

Challenge Question #1

What is an acceptable range for percent recovery?

A. 95-100%

B. 25-30%

C. 80-85%

D. All of the above

Challenge Question #2

During the method validation, dilution integrity was demonstrated for a factor of 10. Which dilution factors applied to the study samples are acceptable when samples are reanalyzed for the reason of ULOQ?

A. 6

B. 10

C. 20

D. Both A and B

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