


Sample Homogeneity, Incurred Sample Repeat Analysis, Data Comparisons and What to Do With It All? - A Case Study



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- Regulatory Backdrop:
 - Repeat Analysis
- Analytical Backdrop:
 - Validation and Sample Analysis
- Case Study:
 - Incurred Sample “pk request” Repeat Analysis
 - Investigation
 - Process and Experiments
 - Final Outcome
- Issues & Recommendations
 - Investigations
 - Incurred Sample Repeats

- Section 7 of the recent Crystal City whitepaper discusses reanalysis of incurred samples.
- Repeats give additional assurance that the method gives reproducible results on samples over time

Analytical Backdrop



- A sensitive, specific, accurate, and reproducible LC/MS/MS assay was validated using a stable label internal standard for the determination of a marketed compound in human plasma.
- The assay validated very well and met all validation guideline requirements
- The validated method was applied for the analysis of 1260 study samples.
- Analysis of study samples proceeded very smoothly with no failed runs.

Validation data



- Accuracy: within 5%
- Precision: within 10%
- Specificity: within 13% (6 lots at LLOQ; accuracy and precision)
- Analyte recovery: 93.6 to 96.6%
- Retention times and peak shape were consistent throughout the validation.

Study Data: Red Flag



- Reanalysis was requested for 8 samples because the pharmacokinetic data did not match historical data.
- 50% of the repeated (n=3) sample values deviated by more than 20% from the original values.
 - Differences as high as 133%.
- Investigation was initiated.

PK Repeat Analysis Results



Sample ID	First Accepted Value (ng/mL)	Repeat Average (ng/mL)	%RSD (Repeats)	% Deviation from 1st Value
1	137	116	6.9	-15.3%
2	45	92	9.47	104%
3	150	115	3.28	-23.3%
4	56.4	86.8	3.2	53.9%
5	112	125	6.48	11.6%
6	38.3	89.2	6.78	133%
7	79.4	71	8.25	-10.6%
8	42.7	66.3	7.65	55.3%

Investigation Initiation



- What is the source of the irreproducible results?
 - Execution?
 - Switched samples
 - Instrument issues
 - Scientist performance of method
 - Method?
 - Metabolite interferences
 - Poor ruggedness
 - ISTD response
 - Samples?
 - Matrix effects
 - Mislabeled

Initial Investigation



- No apparent issues related to above items
- More data required to understand the problem
- Decision in discussion with sponsor
 - Run 10 complete subject profiles (n=3)
 - Concurrent to planning analysis tubes were verified to ensure sufficient volume remaining
- Observation made that sample tubes were “overfilled”
 - Headspace less than 10 mm from cap following initial analysis

- Hypothesis
 - Overfilled tubes + concentration gradient = incomplete mixing resulting in irreproducibility
 - Therefore, reanalysis of 3 subject profiles (56 samples) was performed with **hand mixing/inversion** of each individual sample

Initial Investigation Results (first three profiles)



- Improved pharmacokinetic results
- Excellent within replicate precision
 - Maximum CV < 9%
 - Average CV 3.5%
- Poor correlation to initial results
 - Deviations ranged from 6% to 271%
 - Average deviation of 37%
- Reanalysis halted, consultation with sponsor and investigation expanded

- Positive:
 - Repeats with hand mixing intra-run results were precise & matched historical data
- Negative:
 - Repeat results don't match original
- Conclusion:
 - Our hypothesis is supported but can it be completely proven with data?
- Next Step?
 - Re-assay study with hand mixing OR
 - Get the proof

Above and Beyond



- Small in-house study design to replicate the conditions of sample collection and handling
- 4 volunteers dosed and blood samples collected/plasma harvested per clinical procedure.
 - Plasma from each sample divided into two groups
 - Each group had a storage tube completely filled and partially filled

Above and Beyond



- Study variables:
 - One set of full and partially filled tubes were mixed prior to freezing and analysis
 - One set of full and partially filled tubes were **not** mixed prior to freezing or analysis
 - Analysis of aliquots taken from top and middle of the full tubes and top of partial fill
 - Samples analyzed in triplicate on two separate days by two separate analysts

Results Table Day 1 Analysis: Full Tube/Mixed



Volunteer ID	DAY 1 Triplicate Mean	DAY 1 %CV	%Difference (Top vs Mid)
1 - Top Draw	47.7	0.55	-6.1
1 - Mid Draw	44.8	1.01	
2 - Top Draw	29.3	1.69	-3.8
2 - Mid Draw	28.2	3.01	
3 - Top Draw	37.6	3.19	-4.8
3 - Mid Draw	35.8	5.27	
4 - Top Draw	60.7	4.24	-2.3
4 - Mid Draw	59.3	3.5	

Results Table Day 1 Analysis : Full tube/No Mix



Volunteer ID	DAY 1 Triplicate Mean	DAY 1 %CV	%Difference (Top vs Mid)
1 - Top Draw	34.9	12.3	39.5
1 - Mid Draw	48.7	1.07	
2 - Top Draw	22.3	6.72	30.5
2 - Mid Draw	29.1	17.6	
3 - Top Draw	19.6	8.97	116.8
3 - Mid Draw	42.5	11.0	
4 - Top Draw	23.6	14.8	224.2
4 - Mid Draw	76.5	3.66	

Overall Results – Full Tubes



Volunteer ID	DAY 1 Mean	DAY 2 Mean	% Difference Day 1 vs Day 2	Overall %CV
1 – Mix - top	47.7	48.7	2.1	3.5
1 – Mix - mid	44.8	47.5	5.7	
1 – No Mix - top	34.9	21.5	-62.3	43.3
1 - No Mix – mid	48.7	64.2	24.1	
2 – Mix - top	29.3	29.7	1.3	3.0
2 – Mix - mid	28.2	27.9	-1.1	
2 – No Mix - top	22.3	17.4	-28.2	33.2
2 - No Mix – mid	29.1	37.8	23.0	

Investigation Conclusion



- Ultimate cause of the irreproducibility of repeats = sample in-homogeneity.
 - Mix vs. No Mix & Top vs. Mid aliquot draws
 - Top vs Mid deviation ranged from 31% to 224%
 - Concentration gradient proven
 - %CV significantly higher for unmixed samples vs mixed.
 - Unmixed samples ranged from 33.0% to 62.6%
 - Mixed samples ranged 3.4% to 6.2%
- Decision with sponsor
 - Reanalyze all study samples (n=1) with hand mixing
 - An additional 56 samples (same 3 profiles already assayed) were re-assayed to validate the precision of the study sample results

Reanalysis Conclusion



- All n=1 repeats were acceptable
- Repeat results for 56 samples
 - 100% within 21% of original
 - 95% (3/56) within 20% of original
 - 84% (47/56) within 15% of original

- Compliance issues
 - Documentation required for the investigation/re-analysis
 - Corrective Action, Investigation Report (CAIR)
- Data comparison - original and repeat data sets
 - Statistical models – not available in Watson
- Process issues – SOP's
 - Pk repeats
 - CAIR
 - Incurred sample repeats

- Most are too sensitive – particularly with precise assays
- Recommendation: Similar to QCs
 - 67% of repeat results within $\pm 15\%$ of original
 - Be careful of samples within 20% of LLOQ
 - Be careful of samples within 15% of ULOQ

- Investigations
 - Have SOP's in place
 - Involve Client/QAU
 - Complete documentation
- Repeat Analysis
 - Beware of sample mixing issues
 - Keep process simple – SOP driven
 - Choose something realistic based on the complexity of bioanalysis

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