Welcome to the American College of Toxicology's Webinar Series

Implications for Toxicology/Toxicokinetics When ISR Fails

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Outline

- An Abridged History of ISR
- Current Regulatory Expectations
- ISR Explained
- PK/TK and Study Impact
- Two Case Examples of ISR Failure
- Conclusions and Acknowledgements

Definition of “ISR”

ISR = Incurred Sample Reanalyis/Reproducibility

Repeat analysis of a study sample to assess the analytical method’s ability to reproduce the originally measured result

- Testing is currently required by most regulatory agencies
- Applies to most GLP studies (to be further clarified)
- Can only be conducted on actual study samples
- Timing of testing occurs with main study sample analysis
- Failures, when they occur, require investigation and remediation
- Failures can implicate the entire data set

An Abridged History of ISR

- Health Canada required ISR through the 1990's until 2003 but dropped the requirement
  - Much of their experience was noted in the early FDA discussions
- 2006 AAPS/FDA (Crystal City III) – Arlington, VA and subsequent conference report (2007)
  - FDA reported finding significant result differences on reassay
  - Robust debate but general consensus
  - "How" was left unaddressed
- 2008 AAPS/European Bioanalysis Forum/FDA sponsored workshop Arlington, VA; outcome of workshop is the Fast paper from 2009, JAAPS
  - Much of the "how" recommended
  - Still some unanswered questions

Numerous Position Papers Since 2006

- Incurred Sample Reproducibility: views and recommendations by the European Bioanalysis Forum; Timmerman, Bioanalysis, 1(6) 2009
- Confirmatory Reanalysis of Incurred Bioanalytical Samples; Rocci, The AAPS Journal, 9 (3) 2007
- Incurred sample reanalysis: it's just a matter of good scientific practice; Kelley, Bioanalysis, 3 (9) 2011
- GBC (Global Bioanalysis Consortium, representing FDA, EMA, MHLW, and ANVISA) Repeat Analysis and Incurred Sample Reanalysis harmonization team (A7 HT); The AAPS Journal, 16 (6), 2014
  - Presents numerous recommendations for addressing "how"
  - Some very pragmatic albeit controversial points
    - i.e. 5% of total samples or minimum of 6 per study

Current Regulatory Expectations

US FDA

- 2001 Method Validation Guidance (predates ISR requirement)
- Numerous joint FDA/Industry conferences (Crystal City III-V) and Consensus papers have affirmed the expectation and approach
- 2013 Draft Method Validation Guidance Specifies
  - 7% of total samples will be tested for ISR
  - 2/3 of reassayed samples within <20% difference on reassay (30% for LBA)

EMA - European Medicines Agency

- Guideline on bioanalytical method validation, MEA/CHMP/EWP/192217/2009, Committee for Medicinal Products for Human Use (CHMP), 21 July 2011; WC500109686.pdf
- 10% of all samples if <1000 samples; 5% of all samples if >1000

MHLW - Ministry of Health, Labor and Welfare

- Guideline on Bioanalytical Method Validation in Pharmaceutical (25 July 2013, MHLW, Japan)
- Harmonized with EMA requirements

ISR Explained

- Intra- and inter-subject variability can not be predicted or anticipated with the control matrix used for conducting the method validation
  - Fluctuating endogenous analytes can influence background
  - Circulating metabolites can vary based on differences in metabolism
  - Disease state can alter the condition of the samples (i.e. renally impaired)
  - Protein binding, altered matrix effects, and back-conversion of known and unknown metabolites are possible
  - Sample inhomogeneity
  - Concomitant medications
- ISR Reinforces confidence that the method is valid and reproducible for the intended study samples to which its applied

ISR Explained

The intention of ISR "is to ensure a validated method is able to measure concentrations in incurred samples for any given study both accurately and reproducibly" –
Timmerman/EBF, Bioanalysis 2009

"A well-constructed ISR program should lead to continuous review and improvement practices for the laboratory that is conducting ISR experiments." –
2008 Workshop Report, Fast JAAPS 2009

How is ISR conducted at the Bioanalytical Lab?

Which Studies (for a validated method used on a GLP study)?

- Preclinical/TK studies - once / species / laboratory
- All bioequivalence trials
- First clinical trial in subjects; First patient trial
- First trial in patients with impaired hepatic and/or renal function

How is ISR assessed?

- Conduct on individual samples (no pooling)
- Repeat as per original assessment (i.e. singlet LC/MS, duplicate LBA)
- The number of samples repeated should equal 5–10% of the total sample size
- 2/3 of assessed samples should repeat within 20% or original value (30% LBA)
- Select samples from more subjects (fewer samples/subject) to elicit methodological issues
- One sample near Tmax, one near end of elimination phase
- Conduct on subjects spanning the whole study (take into consideration the dosing schedule)
- Should be conducted early in the study (don't wait for the end of study – short TK studies would be an example exception); first available study in validated matrix
- Reporting is in the study report (not the validation report)
- Requires Bioanalytical labs have an ISR SOP
- Failed ISR must include an investigation
- Any method changes require consideration for applicability to ISR; significant method changes require reinvestigation of ISR (SOP driven)
- Do ISR at each lab (conduct ISR with each application of the method within a different lab)

SOP on ISR – Details

- Detail the method of conducting ISR
- How differences between original and reanalyzed results are computed
- Acceptance criteria will be used
- How an investigation of a failed ISR assessment will be conducted, documented, reported, and archived, and
- Where assessment results will be reported and archived

ISR Investigation (Following an ISR Failure)

- A failed ISR assessment does not immediately invalidate the study
  - it does call for suspension of the bioanalytical portion of the study pending outcome
- Full transparency with the SD and Sponsor is required
- Documentation of ISR planned investigation, results, and conclusions
- Each ISR investigation is specific to the individual case at hand
  - Good scientific practices should be applied at every level
- The end result is a conclusion about the applicability of the assay for the purpose of the study
- If the failure results in an impact to data – impact to study and conclusions regarding use of the data are required
- SOP provides guidance for the investigation

Causes for ISR Failure

Method has passed validation and sample analysis acceptance criteria were met

(per regulatory guidelines)

1. Poor execution of the method
   - Sample homogeneity (i.e. thawing/mixing error)
   - Pipetting technique
   - Sample switching (including injection error)
   - Systematic Analytical Error – i.e. error in stock solution used

   As a quality or process check, it can elucidate embedded issues.
2. Method is unsuitable for the samples to which it is applied

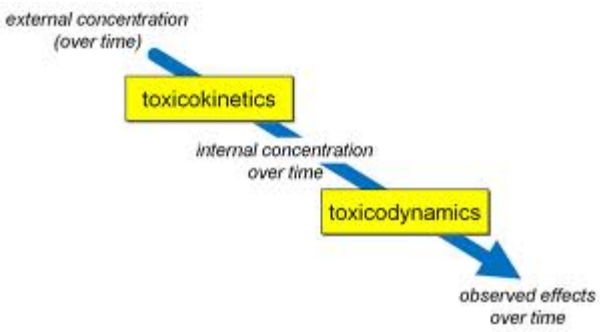
Conducting an ISR investigation

- Stage 1 – review study records for assignable cause
  - Systematic errors in execution
  - Sample sequence and selection correct? (sample switching?)
  - Reagents and reference materials (and solutions thereof)
  - Review all raw data for consistency –
    - Internal standard response, chromatography
  - Consider all data trends – failures clustered by run, subject, time point, dosing group?
- Stage 2 – determine cause and corrective action
  - *Experimental plan documented in advance*
  - Repeat ISR run(s) and one or more initial runs
  - Alter chromatography (i.e. matrix effect) or extraction conditions (unstable metabolites)
  - May require repeat of all study samples with new conditions

Objectives of TK

TK is an integral part of the non-clinical testing program; it should enhance the value of the toxicological data generated

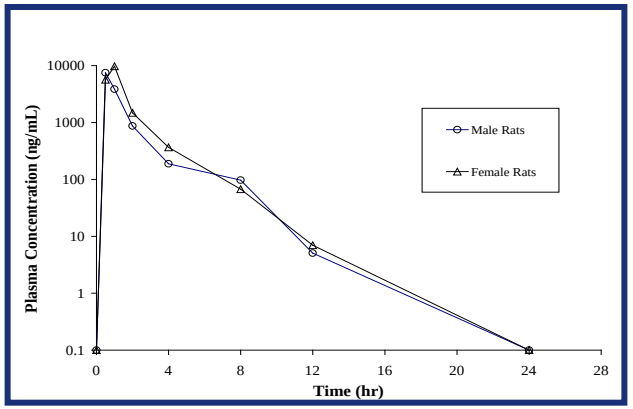
- Sex-difference
- Patterns of drug absorption
- Patterns of drug elimination
- Dose-Systemic Exposure Relationship
  - Dose proportionality
  - Changes in exposure following single- vs. repeated-dosing
  - Steady-state exposure
  - Exposure margins, toxicity-TK relationship
- Support selection of species & treatment regimen nonclinically & clinically
- Facilitate interpretation of toxicological data



TK Guidance

- ICH TK Guideline (S3A)
  - Samples may be from primary studies or from subsequent or satellite studies
  - Not all studies require TK data
  - TK data for a GLP tox study must be conducted according to GLPs
  - Animal exposure will be used to set the allowable limits of human exposure

Must evaluate exposure in animals



Tox / TK implications of failed ISR

- Actions to take for failed ISR (Tox/TK standpoint)
  - Partner closely with SD/PI/Sponsor in assessment of impact
    - Study type, study objective(s)?
    - Previous ISRs conducted?
    - How do data compare to previous study results (if available)
    - Magnitude of bias? Assess overall impact on quantitation?
      - Semi-quantitative PK/TK data is still better than no data at all…
      - With increased bias comes increased risk of making the wrong decision; mitigate accordingly
    - Can other analytes on study be used as an exposure endpoint?
    - Can a PD/TD marker be used as an exposure surrogate?
    - Presence of ADA as an exposure surrogate for biotherapeutics?
  - Perform PK/TK analysis but caution interpretation
  - Case-by-case decision making to provide sufficient information for risk & safety assessment
  - Be fully transparent & describe ISR investigation
  - Update compliance statement accordingly

Case I – Background

- Assay includes TK for parent and glucuronide metabolite
- Known circulating but unquantitated metabolites exist
  - profiling was conducted for early non-GLP studies
- First review of ISR results showed ~50% passing rate for parent and metabolite; Parent shows slight trend for more variability in higher dose groups; metabolite results shows no trend
- ISR results originating from one original analysis (Batch #6) showed higher percentage of failures with some samples grossly out of range (>100%).
- Internal standard (IS) performance suspected for the O-glucuronide metabolite – investigate in parallel

Case I – Investigation Plan

- Co-developed with the SD and Sponsor – fully documented all observations in advance with justification for the selected plan
- Conduct duplicate reanalysis of a subset of the original ISR samples (include passing and not passing samples)
- Use this data to elucidate if the errors occurred in the original analysis or ISR assay
- If original, repeat impacted batches; if ISR, develop an additional plan of action and/or assess impact of ISR failure

ISR Investigation Results - Glucuronide Metabolite

						OR // OrISR	R1 //R2	OR // MnR12	OrISR //MnR12
Sample Name	OR	OrISR	R1	R2	Mean R1/R2				
4830 2 Plasma-1 Day 27 2h	5.23	3.24	2.93	3.75	3.34	46.99	-24.55	44.11	-3.04
4836 2 Plasma-1 Day 27 12h	4.64	5.00	3.41	4.04	3.73	-7.47	-16.91	21.88	29.23
4836 2 Plasma-1 Day 6 1h	5.97	5.15	5.54	5.96	5.75	14.75	-7.30	3.75	-11.01
4836 2 Plasma-1 Day 6 2h	3.44	3.05	3.21	3.37	3.29	12.02	-4.86	4.46	-7.57
4854 2 Plasma-1 Day 6 1h	4.39	3.99	3.55	3.84	3.70	9.55	-7.85	17.19	7.68
4838 3 Plasma-1 Day 6 1h	58.50	51.50	51.50	54.90	53.20	12.73	-6.39	9.49	-3.25
4838 3 Plasma-1 Day 6 2h	41.70	38.30	37.10	39.20	38.15	8.50	-5.50	8.89	0.39
4838 3 Plasma-1 Day 0 4h	9.72	9.63	10.30	8.41	9.36	0.93	20.20	3.83	2.90
4835 4 Plasma-1 Day 0 2h	99.7	115.0	107.0	109.0	108.0	-14.25	-1.85	-7.99	6.28
4835 4 Plasma-1 Day 0 4h	18.0	23.1	20.8	20.9	20.9	-24.82	-0.48	-14.67	10.24
4839 4 Plasma-1 Day 27 4h	56.0	63.3	60.1	61.5	60.8	-12.24	-2.30	-8.22	4.03
4839 4 Plasma-1 Day 6 1h	14.2	15.2	14.9	15.2	15.1	-6.80	-1.99	-5.81	0.99
4839 4 Plasma-1 Day 6 4h	153.0	180.0	163.0	166.0	164.5	-16.22	-1.82	-7.24	9.00
4839 4 Plasma-1 Day 0 2h	125.0	149.0	131.0	133.0	132.0	-17.52	-1.52	-5.45	12.10
4839 4 Plasma-1 Day 0 4h	18.4	19.6	21.4	22.7	22.1	-6.32	-5.90	-18.05	-11.76
4852 4 Plasma-1 Day 27 8h	38.2	42.8	38.2	38.8	38.5	-11.36	-1.56	-0.78	10.58
4852 4 Plasma-1 Day 0 24h	14.9	16.3	15.0	15.3	15.2	-8.97	-1.98	-1.66	7.31
4852 4 Plasma-1 Day 0 2h	134.0	131.0	124.0	131.0	127.5	2.26	-5.49	4.97	2.71
4856 4 Plasma-1 Day 27 4h	148.0	165.0	143.0	144.0	143.5	-10.86	-0.70	3.09	13.94
4856 4 Plasma-1 Day 27 8h	19.9	22.4	20.6	23.3	22.0	-11.82	-12.30	-9.80	2.03
4859 4 Plasma-1 Day 27 12h	9.6	11.2	10.2	10.3	10.3	-15.38	-0.98	-6.55	8.86
4859 4 Plasma-1 Day 6 8h	47.4	51.2	47.6	52.4	50.0	-7.71	-9.60	-5.34	2.37
4861 4 Plasma-1 Day 6 4h	155.0	157.0	171.0	150.0	160.5	-1.28	13.08	-3.49	-2.20
					More than 20%	5	3	4	1
					More than 50%	2	0	0	1
Total Samples	67				Percent Pass	92.5	n/a	94.0	98.5

- When IS and Batch #6 results eliminated, all results passing
- Duplicate repeats of Batch #6 were in line w/ ISR results

ISR Investigation Results – Parent (TA)

Sample Name	OR	OrISR	R1	R2	Mean R1/R2	OR // OrISR	R1 //R2	OR // MnR12	OrISR //MnR12
4830 2 Plasma-1 Day 27 2h	220	242	189	194	191.50	-9.52	-2.61	13.85	23.30
4836 2 Plasma-1 Day 27 12h	221	222	182	200	191.00	-0.45	-9.42	14.56	15.01
4836 2 Plasma-1 Day 6 1h	88	90.6	82.8	83.8	83.30	-2.91	-1.20	5.49	8.40
4836 2 Plasma-1 Day 6 2h	112	140	102	107	104.50	-22.22	-4.78	6.93	29.04
4836 2 Plasma-1 Day 0 1h	130	107	117	119	118.00	19.41	-1.69	9.68	-9.78
4854 2 Plasma-1 Day 6 1h	101	144	99.1	100	99.55	-35.10	-0.90	1.45	36.50
4829 4 Plasma-1 Day 6 12h	840	672	471	490	480.50	22.22	-3.95	54.45	33.23
4834 4 Plasma-1 Day 27 2h	2020	3070	1690	1770	1730.00	-41.26	-4.62	15.47	55.83
4834 4 Plasma-1 Day 6 2h	2990	2820	1950	2000	1975.00	5.85	-2.53	40.89	35.25
4834 4 Plasma-1 Day 6 4h	1840	1880	1200	1240	1220.00	-2.15	-3.28	40.52	42.58
4834 4 Plasma-1 Day 0 1h	3210	2040	1520	1620	1570.00	44.57	-6.37	68.62	26.04
4835 4 Plasma-1 Day 27 2h	2570	4320	2450	2670	2560.00	-50.80	-8.59	0.39	51.16
4835 4 Plasma-1 Day 6 2h	1510	2140	1450	1480	1465.00	-34.52	-2.05	3.03	37.45
4835 4 Plasma-1 Day 0 2h	2340	3440	1890	1970	1930.00	-38.06	-4.15	19.20	56.24
4835 4 Plasma-1 Day 0 4h	619	836	591	497	544.00	-29.83	17.28	12.90	42.32
4839 4 Plasma-1 Day 27 4h	1300	1650	1230	1260	1245.00	-23.73	-2.41	4.32	27.98
4839 4 Plasma-1 Day 6 1h	1480	2870	1340	1530	1435.00	-63.91	-13.24	3.09	66.67
4839 4 Plasma-1 Day 0 4h	544	934	633	642	637.50	-52.77	-1.41	-15.83	37.73
4852 4 Plasma-1 Day 27 8h	410	334	281	292	286.50	20.43	-3.84	35.46	15.31
4852 4 Plasma-1 Day 0 24h	1640	1470	1030	1080	1055.00	10.93	-4.74	43.41	32.87
4852 4 Plasma-1 Day 0 2h	3530	2870	2200	2310	2255.00	20.63	-4.88	44.08	24.00
4856 4 Plasma-1 Day 27 4h	2250	1700	1380	1450	1415.00	27.85	-4.95	45.57	18.30
4856 4 Plasma-1 Day 27 8h	1170	1130	994	1000	997.00	3.48	-0.60	15.97	12.51
4859 4 Plasma-1 Day 27 12h	332	285	264	269	266.50	15.24	-1.88	21.89	6.71
4859 4 Plasma-1 Day 6 8h	1530	1200	1010	1030	1020.00	24.18	-1.96	40.00	16.22
4861 4 Plasma-1 Day 6 4h	2900	1710	2370	1680	2025.00	51.63	34.07	35.53	-16.87
Total Samples	69				More than 20%	26	1	31	32
R1 // R2 count	50				Percent Pass	62.3	98.0	55.1	53.6

Low Dose Group

High Dose Group

- While duplicate R1/R2 match, the results match neither original (OR) or Mean R1/R2
  - Approximately 50% repeat pass rate
- Indicates a more persistent assay issue which would require assay redevelopment

Case I – Summary

- Original ISR results shows <50% passing initially, parent and metabolite
- Identified Batch #6 had original assay issues
  - confirmed by duplicate repeat after first ISR
- Internal standard (IS) performance for glucuronide metabolite ultimately was deleterious to assay performance as revealed through ISR
  - Drop IS for all validation and SA runs
- Performance of the Parent TA was unable to be improved without redeveloping the assay
  - Would require use of incurred study samples
  - Full revalidation would be required

Case I – Study Impact

- TK parameters were calculated for parent and metabolite
  - Parent data was contexted as not having been assessed with required reproducibility in the bioassay
  - Therefore inherent limitations in quantitative value of the TK parameters exist – qualitatively parent exposure was demonstrated
  - Metabolite data requires no contexting – all TK parameters reported

OVERALL – exposure and TK were adequately assessed particularly in view of the metabolite data and with limited additional information coming from parent data.

However - as safety margins (i.e. for FIH/FIM) rely on parent data (as well as active metabolites) – use of this data could require further considerations such as use of a more conservative safety window for entry dose setting (FIH/FIM)…

Case Example II – Whole Blood Analysis

- TA undergoes rapid esterase degradation *in vivo*
- A method to stabilize was provided by Sponsor – using crashed whole blood
- Sample collection was optimized to preserve TA and metabolite (both measured for TK) – quench esterase activity with acid and organic
- "Crash" sampling procedure specified in protocol
  - Whole blood was collected into the pre-weighed tubes without anticoagulant;. Each tube with sample was weighed
  - Deliver a 10% SDS/ascorbic acid/HCl and 1% FA in ACN solution was immediately added to the blood in the proportion of 1:1:1 (w:v:v).
  - Vortex-mixed and flash freeze in an ethanol/dry ice bath
  - Stored frozen at approximately -70˚C
  - Record all times
- All matrix used for STD/QC preparation used crashed whole blood prepared in this manner

Case II – Rapid Whole Blood Degradation

- Validation testing showed sharp drop in TA concentrations in whole blood
- However - Stability of TA was demonstrated for up to 5 hours in “crashed” whole blood

TA in whole bood

- no stabilizers
- values normalized to T0
- data from validation

Storage Duration (minutes)	Area Ratio	Mean Area Ratio	RSD (%)	Percent Time Zero
T0	66E-01 48E-01 78E-01	4.64E-01	3.2	NA
1.00	03E-01 93E-01 57E-01	2.84E-01	8.5	**61**
2.00	00E-01 17E-02 03E-01	9.84E-02	6.1	**35**
5.00	48E-02	6.81E-02	4.7	**24**

Case II - ISR Results Failure

- Repeat concentrations trend high both TA and M1
- 55% and 65% pass rate (66% overall is required)

failure TA and M1
failure TA only
failure M1

Mandatory Repeat Report Concentrations of TA in Plasma (ng/mL) (Original - Repeat)/(Mean) x 100 = %RE 20 Samples: 9 Flagged (45.0 %); 0 NC(0.00 %)						Mandatory Repeat Report Concentrations of M1 in Plasma (ng/mL) (Original - Repeat)/(Mean) x 100 = %RE 20 Samples: 7 Flagged (35.0 %); 1 NC(5.00 %)					
Final Analysis Run ID	Final Original Concentration	Repeat Run ID	Repeat Concentration	%RE	Flag	Final Analysis	Final Original	Repeat Run ID	Repeat Concentration	%RE	Flag
1	79.5743	5	161.209	-67.8	>30	1	18610.3	5	27617.3	-39	>30
1	55.8191	5	75.2487	-29.6		1	30411.6	5	33789.1	-10.5	
2	60.2813	5	40.3002	39.7	>30	2	41341.5	5	33280.7	21.6	
2	12.9922	5	13.4933	-3.8		2	30659.8	5	29555.7	3.7	
2	9.66544	5	11.3651	-16.2		2	23023.5	5	28528.1	-21.4	
3	6.03233	5	5.88913	2.4		3	24540	5	20825.1	16.4	
1	93.9227	5	120.832	-25.1		1	38173.3	5	47288.1	-21.3	
1	23.1797	5	36.5239	-44.7	>30	1	40127.4	5	56093.6	-33.2	>30
1	72.8233	5	99.4589	-30.9	>30	1	52414.4	5	76404.6	-37.2	>30
2	16.2882	5	13.6186	17.9		2	69660.9	5	64970.3	7	
2	79.5704	5	113.26	-34.9	>30	2	53396.7	5	59183.1	-10.3	
2	66.2352	5	87.7801	-28		2	75843.9	5	76584.6	-1	
3	21.8994	5	21.3247	2.7		3	29722.7	5	21501.2	32.1	>30
3	7.46461	5	14.4841	-64	>30	3	51807.8	5	78783.9	-41.3	>30
3	16.888	5	59.6335	-112	>30	3	38201.1	5	62567.3	-48.4	>30
3	20.8516	5	19.3835	7.3		3	97749.8	5	92086.4	6	
3	7.83437	5	8.64081	-9.8		3	92571.2	5	90565.6	2.2	
3	15.5617	5	14.7758	5.2		3	96378.8	5	96990.5	-0.6	
1	81.9238	5	201.509	-84.4	>30	1	45519.5	5	108969	-82.1	>30
1	24.3632	5	48.4427	-66.1	>30	1	80717.8	5	ALQ>(120000)	*	N.C.

Case II - Investigation Conducted

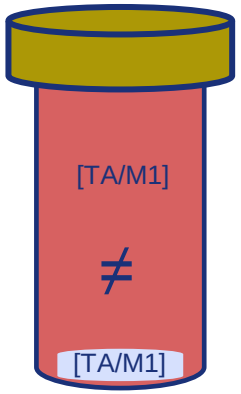
- Informed and engaged SD and Sponsor!!
- Focus on hypothesis that equilibrium between pelleted matrix material (present from crash and observed in all samples) was dynamic and perturbed by handling samples
  - The pellet and ACN crash solvent (with stabilizers) represents a non-homogeneous sample
  - The pellet/ACN mixture is likely subject to changes in equilibrium that would give rise to changing concentrations
- Document Investigation Plan (each part) before conducting – included in the study records
- Due to the unusual matrix, some differences in QCs vs samples were noted
  - The pellet was not stored with QCs
  - TA/M1 was added to the supernatant of crashed whole blood

Case II – Investigation
Focus on Pellet/Supernatant Equilibrium

- Could TA and M1 be distributed unevenly in pellet?
- Manipulation of sample could perturb pellet and release additional TA/M1 into supernatent

Part 1

- QCs - spike TA/M1 into whole blood then crash
- Compare F/T with and w/o pellet
- Stress by doing n=3 F/T cycles



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Part 2

- QCs - spike before whole blood crash
- Further disrupt pellets w/ sonication (ice/RT)
- Spike after crash and freeze (pellet kept in contact w/ supernatant)
- Repeat a subset of the ISR samples

Results of Investigation Part 1

Results of Investigation Analysis - Part I				
Sample	**TA Conc**	**% of first value**	**M1 Conc**	**% of first value**
Tube 1 - Thaw 1	14.6	--	14400	--
Tube 1 - Thaw 2	16.2	111.0	14100	97.9
Tube 1 - Thaw 3	16.4	112.3	14100	97.9
	RSD:	*6.3*	*RSD:*	*1.2*
Tube 2 - Thaw 1	15.6	--	13700	--
Tube 2 - Thaw 2	15.0	96.2	13000	94.9
Tube 2 - Thaw 3	16.0	102.6	15500	113.1
	RSD:	*3.2*	*RSD:*	*9.2*
Tube 3 - Time 0	22.8	--	17000	--
Tube 3 - Thaw 1	22.4	98.2	18200	107.1
Tube 3 - Thaw 2	22.2	97.4	19800	116.5
Tube 3 - Thaw 3	21.7	95.2	18000	105.9
	RSD:	*1.6*	*RSD:*	*5.3*
Tube 4 - Time 0	26.5	--	18600	--
Tube 4 - Thaw 1	26.1	98.5	18700	100.5
Tube 4 - Thaw 2	25.7	97.0	16600	89.2
Tube 4 - Thaw 3	26.0	98.1	17300	93.0
	RSD:	*0.8*	*RSD:*	*6.1*

Pellet remained in sample through freeze/thaw

Pellet removed prior to freeze/thaw

- Results failed to demonstrate the effect observed in study samples
  - No change in TA/M1 with added freeze/thaws
- Accuracy of TA/M1 added before crash low (20-50%) as compared to spike post » confirms binding to pellet

Investigation - Part 2

- 2a. Force more disruption of pellet (break up with sonication)
  - Cycle [sonicate, hold at bench top, remove aliquot] repeat
  - Ice bath vs RT compared
    - Result: no change from Part 1 – further vigorous pellet manipulation doesn't replicate the ISR SA data
- 2b. Spike post crash while leaving pellet in tube
  - Result: Significant binding to the pellet – 32% and 44% accuracy relative to nominal
- 2c. Repeat a subset of the ISR samples – those showing >30% difference
  - Result: Duplicate repeat concentrations are consistent with first ISR. Results are stable with RSD of all repeat results <10%.

ISR Case II – Results from Part 2

- No evidence of increased or changing concentration with further pellet disruption on additional stress (sonication and bench top, RT/ice)
- Significant evidence of TA/M1 loss into the pelleted material; up to 85% loss for TA, 70% for M1
- Repeat of ISR (duplicate) shows stable concentrations with close agreement in R1/R2 and original ISR result
- Investigations could not reproduce the phenomenon observed with ISR samples
- ISR results were accepted by the client for further discussion of impact within the study data

Case II – Study Impact

Study Impact statement

- TK parameters C_{max}, T_{max}, T_{last} were reported for TA and M1
- The variability in the TA (due to rapid degradation in whole blood) is noted with the TK results; M1 data is not similarly impacted
- The ISR data suggests increased variability in values due to co-precipitation of TA and M1 with the pellet;
  - *Therefore concentrations reported in the initial analysis represent a lower level than actual levels in vivo.*
- Based on these data, sufficient exposure was observed to achieve study objectives

Ultimately, with transparency in the limitations for its use, the TK data may be able to be considered for fulfilling study (and regulatory) objectives.

Conclusion

- ISR provides additional data to improve confidence in the reliability / reproducibility of a validated method for nonclinical & clinical study samples
- If PK/TK determination is an outcome of a study, then an ISR assessment should be considered in the BioAC portion of the study
- If ISR data fails to meet the *a priori* acceptance criteria, root cause investigation should be conducted for the BioAC method & necessary measures should be taken by considering the potential impact on study sample analysis
- A failed ISR assessment does not immediately invalidate the entire study (or even the PK/TK phase), but it does call for suspension of the BioAC portion of the study until an investigation is completed, documented, & appropriate follow-up actions are in place

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Elizabeth Groeber is a graduate of The Ohio State University with a Ph.D. in Analytical Chemistry. Following her graduation, she joined a contract research organization in Austin, Texas, CEDRA Corporation (now World Wide Clinical Trials) performing HPLC/MS/MS analysis of small molecules for GLP/21 CRF Part 58 compliant studies. While there she held several roles from method developer to Principal Investigator and founded an internal group performing GLP certification of reference materials used as part of conducting regulatory studies. In 2004 she joined Pfizer, Inc.'s bioanalytical group in the PDM (Pharmacokinetics, Dynamics, and Metabolism) department, in Groton, CT. There she focused on novel approaches for increasing throughput (i.e. small and solid core particle stationary phases) in a regulated environment. In 2008 she transferred into a bioanalytical role focused on biomarkers supporting the neuroscience and cardiovascular teams in both discovery and development with assays including peptide and protein quantitation by LC/MS/MS. With rapidly evolving changes in the Pfizer pipeline, she also contributed to biotherapeutic analysis of ADCs (antibody drug conjugates) and developed internal strategies for monitoring the ADCs in metabolism studies. In 2013 she moved into the Director of Bioanalytical Chemistry role at WIL Research, Ashland, OH. In this, her current role, she oversees the HTPK group conducting discovery stage small molecule and peptide analysis and the regulated bioanalytical group, focused on small molecules, biotherapeutics, and biomarkers for pharmaceutical and agricultural products. Elizabeth's (Beth's) total career spans 18 years in the bioanalytical field.

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Bio: Dr. Andy Vick is currently a member of the senior leadership team at WIL Research, serving as the Sr. Director of Analytical Services. In this role, he oversees the scientific, operational, and financial aspects of the analytical, formulations, bioanalytical, drug metabolism and pharmacokinetic services for sites in the United States. Prior to joining WIL Research, he was Executive Vice President and Sr. Director of Pharmacokinetics, Dynamics and Metabolism (PDM) at Seventh Wave Laboratories where he supported strategic operations as well as scientific efforts across PDM especially in early stage drug development. In addition, Andy has been the Scientific Director of the BioPharma Services Division of Millipore, Principal Scientist within the Drug Disposition and Toxicology department of Eli Lilly and Company, and a preclinical scientist at Biogen. In these roles, Andy has contributed to the design, conduct, and interpretation of preclinical and clinical testing strategies for both small organic and biotherapeutic molecules across a variety of therapeutic indications and stages of development.

Andy is currently a Board Member and Chair of the Biotech special interest group of the International Pharmaceutical Federation. In addition, he has been an active member of AAPS, currently serving on the Executive Committee of the AAPS Foundation and AAPS's Executive Council as Member-At-Large. He also sits on the Board of The Ohio State University's College of Pharmacy. Andy received his BS (Zoology) and PhD (Pharmaceutical Chemistry) from The Ohio State University.