QUOTIENT BIORESEARCH

Challenges of validating small molecule LC-MS/MS biomarker methods

Answers Through Innovation

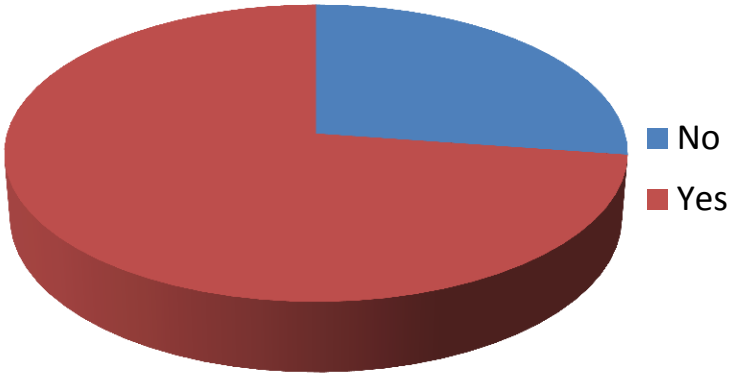
Introduction

- Bioanalytical classification
- White paper guidance
- Risk based approach to qualification/validation
- Setting acceptance criteria
- Endogenous assay validation – specific issues
- Surrogate matrix calibration
- Case study

Illustrated with answers from recent GCC questionnaire

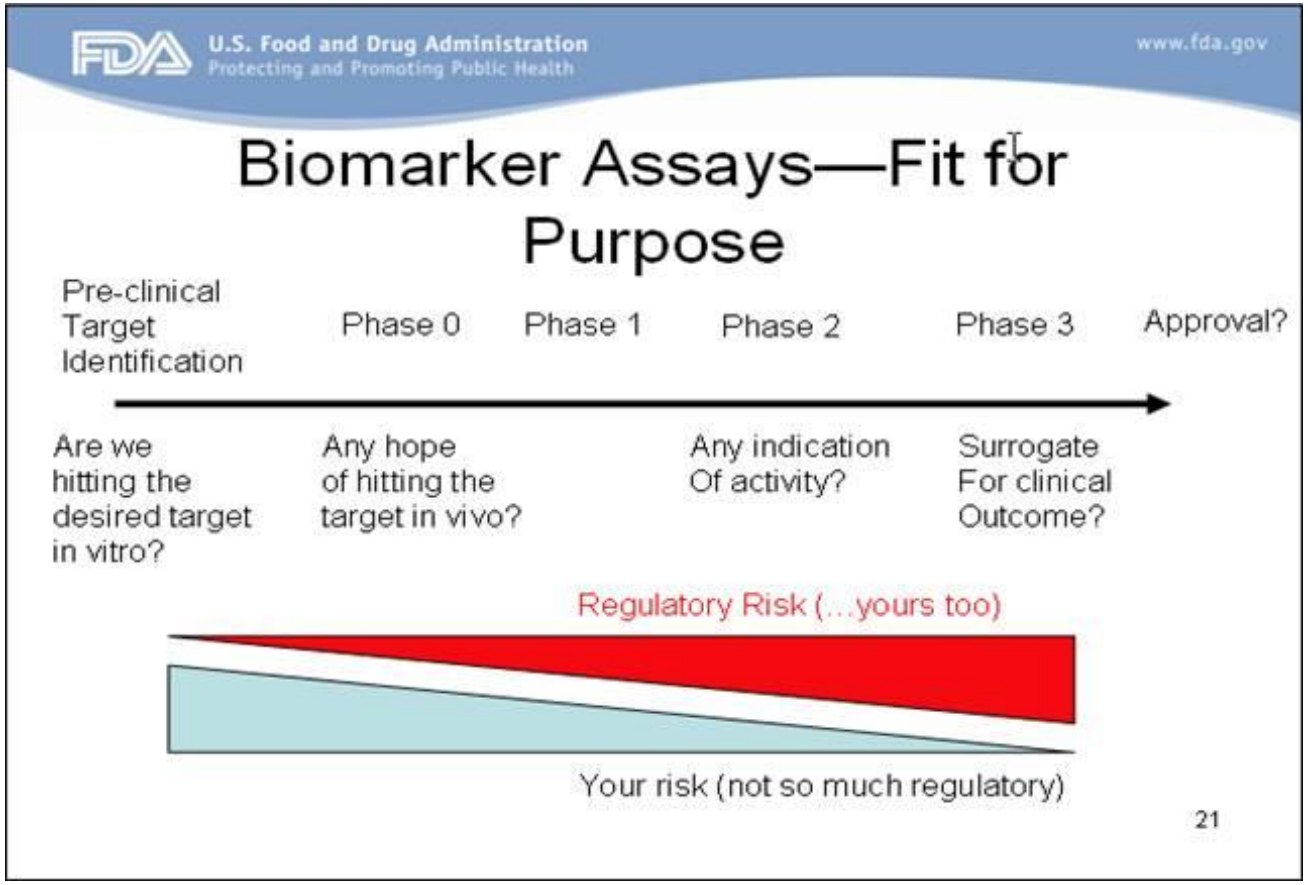
GCC Questionnaire - Classification

Does your organisation apply different classification to biomarkers based on study endpoint and what are they?



- Primary Differentiation for Bioanalysis:- Exploratory versus Study-endpoint

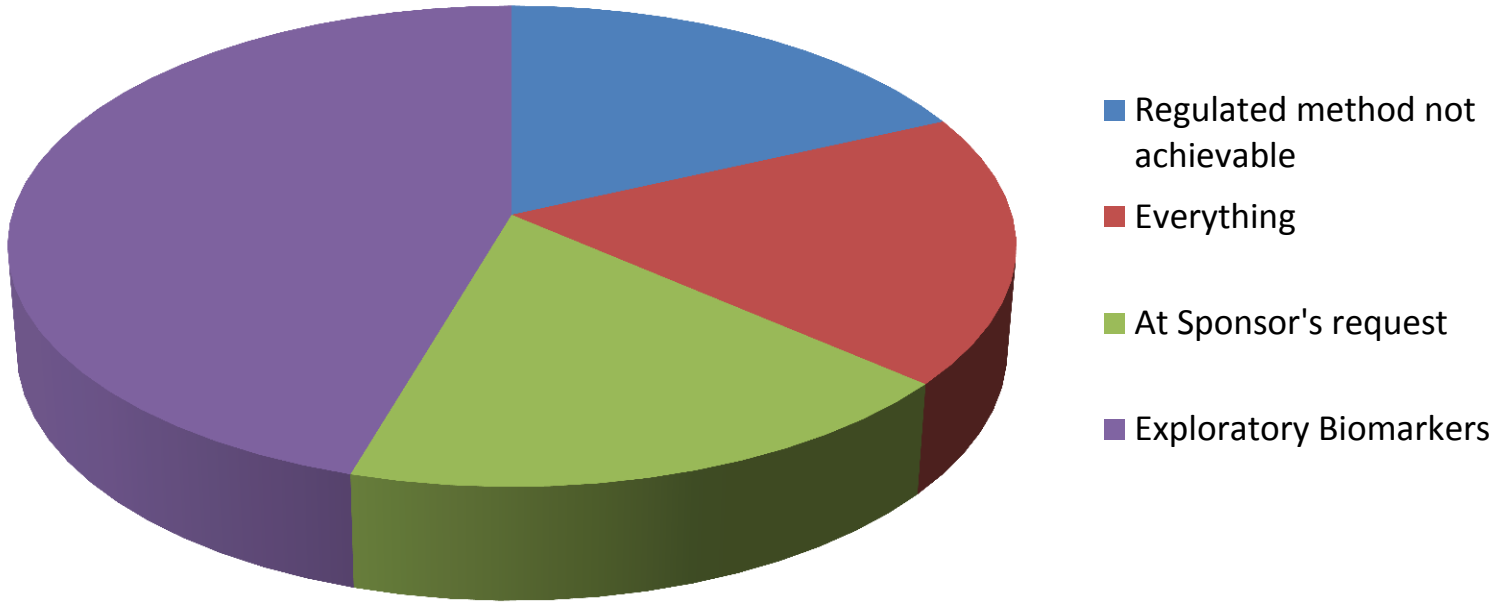
Validation vs Qualification – Risk Approach



Brian Booth - Reid Bioanalytical Forum July 2009

GCC Questionnaire – Method Qualification

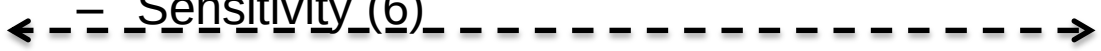
When would your organization apply a “fit-for-purpose” qualification to a biomarker method?



GCC Questionnaire – Method Qualification

What parameters would you include in a "fit-for-purpose" qualification of a biomarker

- **Small molecule (/10 replies)**
  - Calibration (10)
  - P&A (10)
  - Selectivity (9)
  - Matrix Effects (7), Parallelism (3)
  - Storage stability (7)
  - Sensitivity (6)



Minimum?

  - Linearity of dilution (4)
  - Recovery (4)
  - Others (reference ranges, carry-over)

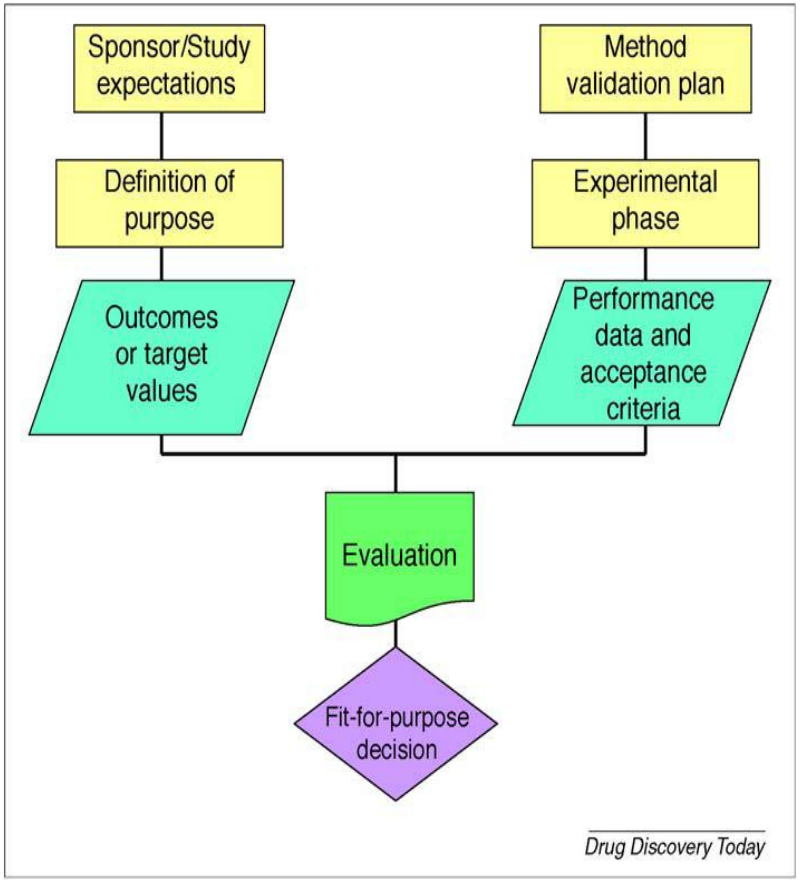
GCC Questionnaire – White Paper Guidance

What industry reference documents do you refer to for biomarker "qualification/validation"?

- FDA/EMA guidance
- Lee *et al.* (2006) & (2009)
- Chau *et al.* (2008)
- Cummings *et al.* (2010)
- Valentin *et al.* (2011)
- CLSI guidelines (formerly NCCLS)

Fit-for-Purpose Validation – Flow Chart

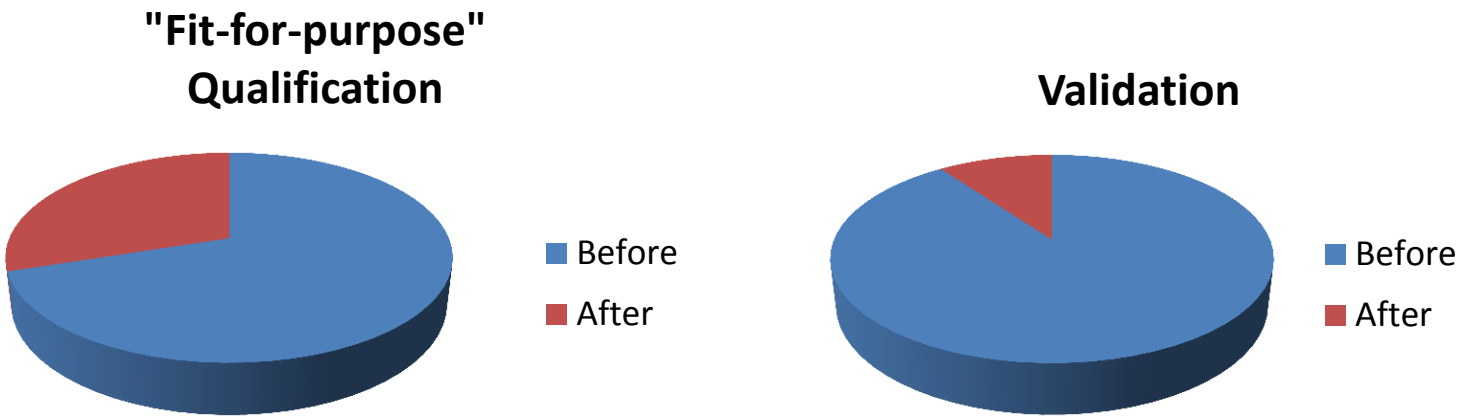
- Establish expectations of sponsor or scientific goal
- Define the purpose of the assay in terms of target values and acceptance limits
- Characterise performance of method by experimentation



Cummings *et al.* Drug Discovery Today (2010)

GCC Questionnaire – Acceptance Criteria

Do you set acceptance criteria before or after the method "qualification/validation" for biomarker methods?



- ICON – Acceptance criteria for QCs during sample analysis is statistically linked to the performance of the method at validation using a confidence limit approach

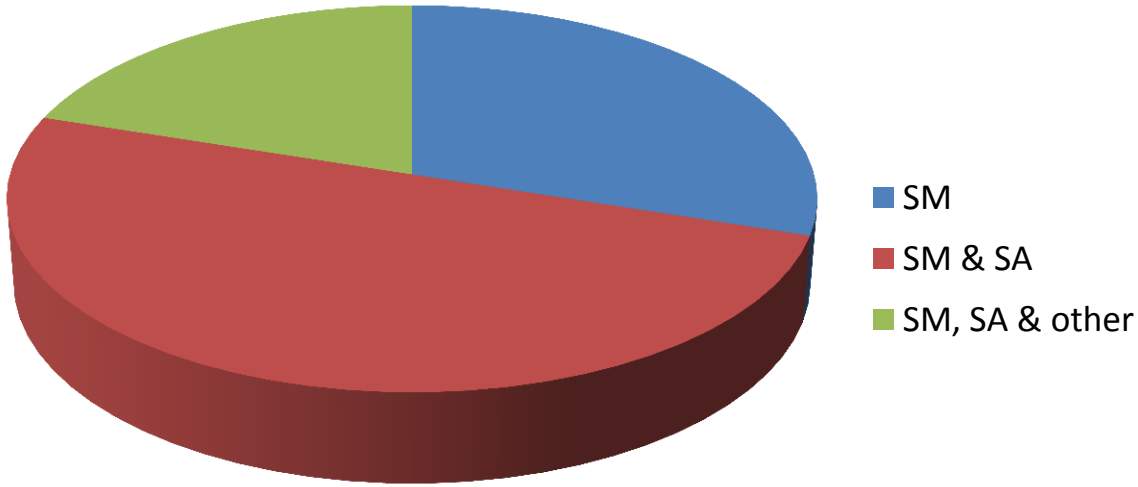
Endogenous Assay Validation – Specific Issues

- The issue of endogenous assay validation is not well described in the regulations for small molecules
- Different approaches include:-
  - surrogate analyte
  - standard addition and extrapolation
  - surrogate matrix
- Choice of surrogate matrix
  - analyte free (hooray!)
  - stripped
  - synthetic

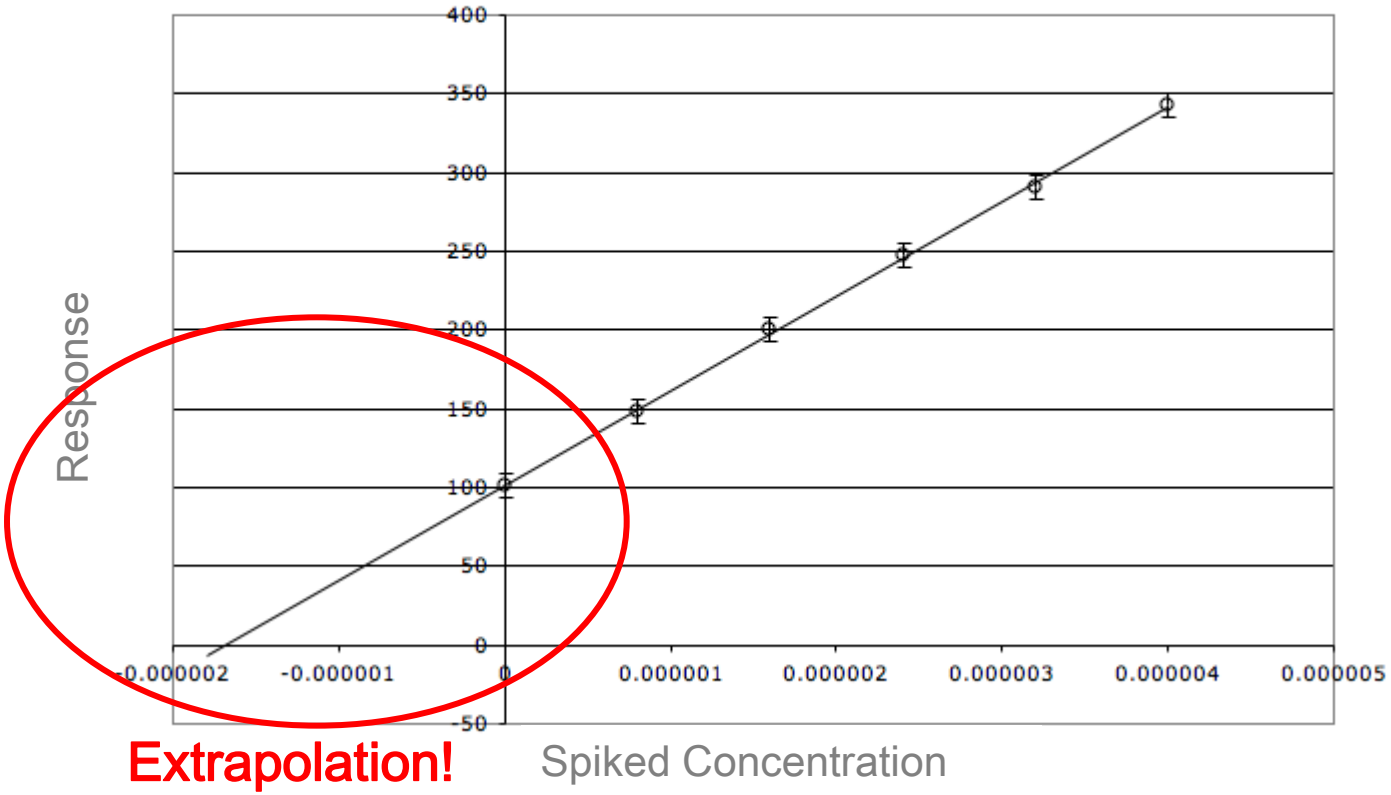
Beware matrix effects!

GCC Questionnaire – Method Calibration

For small molecule biomarker methods, do you use a surrogate matrix (SM), standard addition (SA) or some other approach (other)?



Standard Addition Calibration



Standard Addition Calibration

- Advantages
  - Matrix match calibration stds and samples
- Disdvantages
  - Difficult to estimate the LLOQ
  - Quantification software not always designed to handle standard addition calibration
  - Difficult to construct standard addition calibration where endogenous concentrations are high
  - bioanalytical regulations discourage extrapolation of calibration

Surrogate Matrix Calibration

- Advantages
  - Conventional quantitative processing of calibration
  - LLOQ instrument response can be measured directly (albeit in surrogate matrix)
  - No extrapolation of calibration
- Disadvantages
  - High probability of matrix effects

Quotient approach is surrogate matrix calibration for small molecule LC-MS/MS applications

Method Development – Batch Design

- Calibrate in surrogate matrix
- Use mix of matrix, diluted matrix and surrogate matrix QCs
  - Medium QC (undiluted pooled control matrix)
  - High QC (spiked control matrix)
  - Low QC (diluted control matrix ~x3 LLOQ)
  - LLOQ QC (spiked surrogate matrix)
- Minimise any potential matrix effects during method development
- With LC-MS/MS, SIL IS greatly increases the chances of success
- Check %RE of diluted matrix during method development

Method Development – P&A

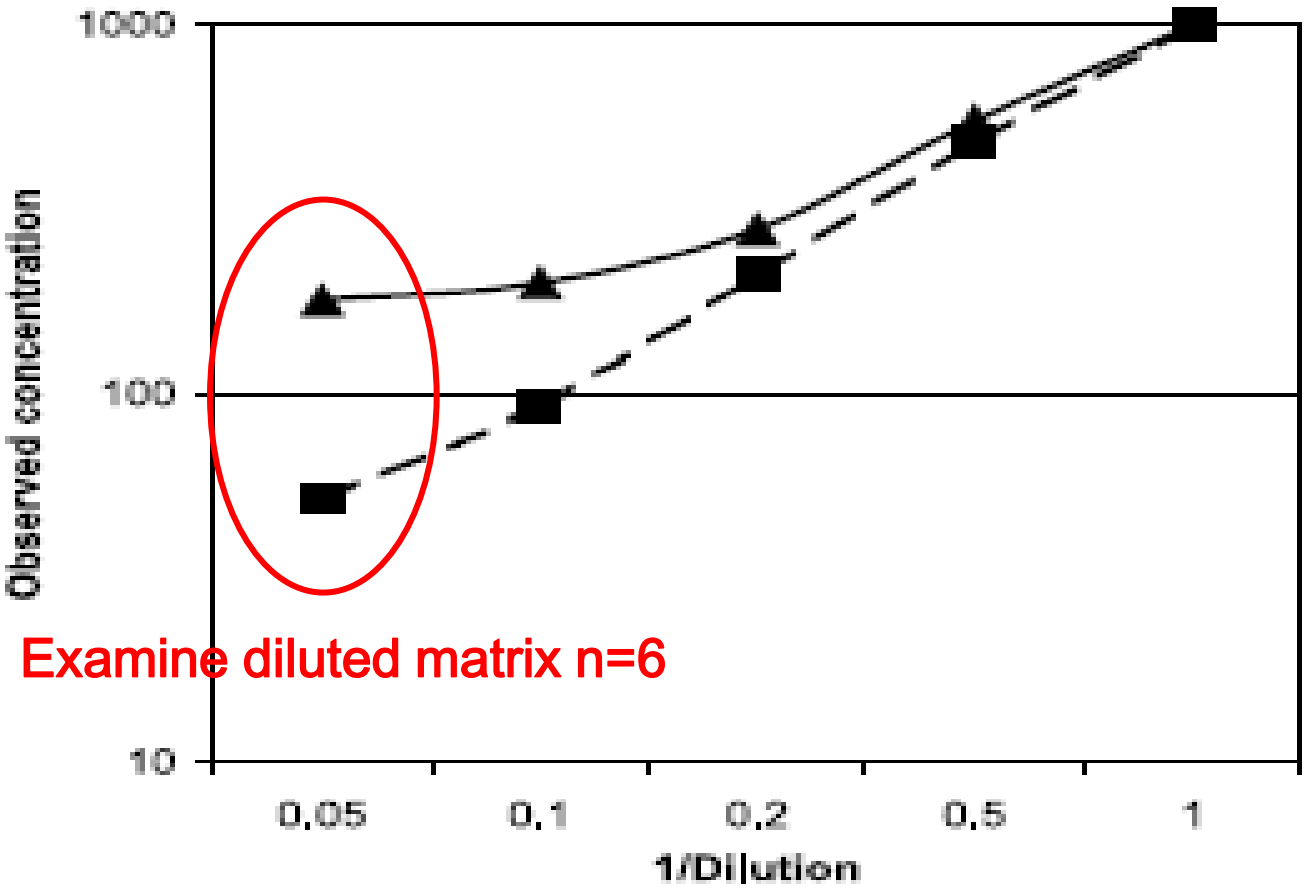
Androstendione in human urine

Measured endogenous concentration in control urine

QC ID Concentration	LLOQ 0.200 ng/mL	QC LOW 0.496 ng/mL	QC MED 12.4 ng/mL	QC HIGH 132 ng/mL
	Surrogate Matrix	Diluted Matrix	Matrix	Spiked Matrix
Replicate 1	0.218	0.516	12.6	128
2	*0.282	0.518	12.5	128
3	0.212	0.524	11.9	128
4	0.16	~0.604	12.1	130
5	0.173	0.558	12.3	129
6	0.187	~0.620	12.1	134
7			12.5	
8			12.4	
9			13	
10			12.3	
Intrarun Mean	0.19	0.557	12.4	130
Intrarun SD	0.0248	0.0458	0.309	2.35
Intrarun %CV	13.1	8.2	2.5	1.8
Intrarun %RE	-5	12.3	0	-1.5
n	5	6	10	6

bias would probably indicate uncorrected matrix effects or differential recovery

Method Validation - Parallelism



•Fit--for-purpose method development and validation for successful biomarker measurement. J. W. Lee *et al*., *Pharm. Res.* 23(2):312-328 (2006).

Method Validation – Matrix Effects

Etiocholanolone in urine

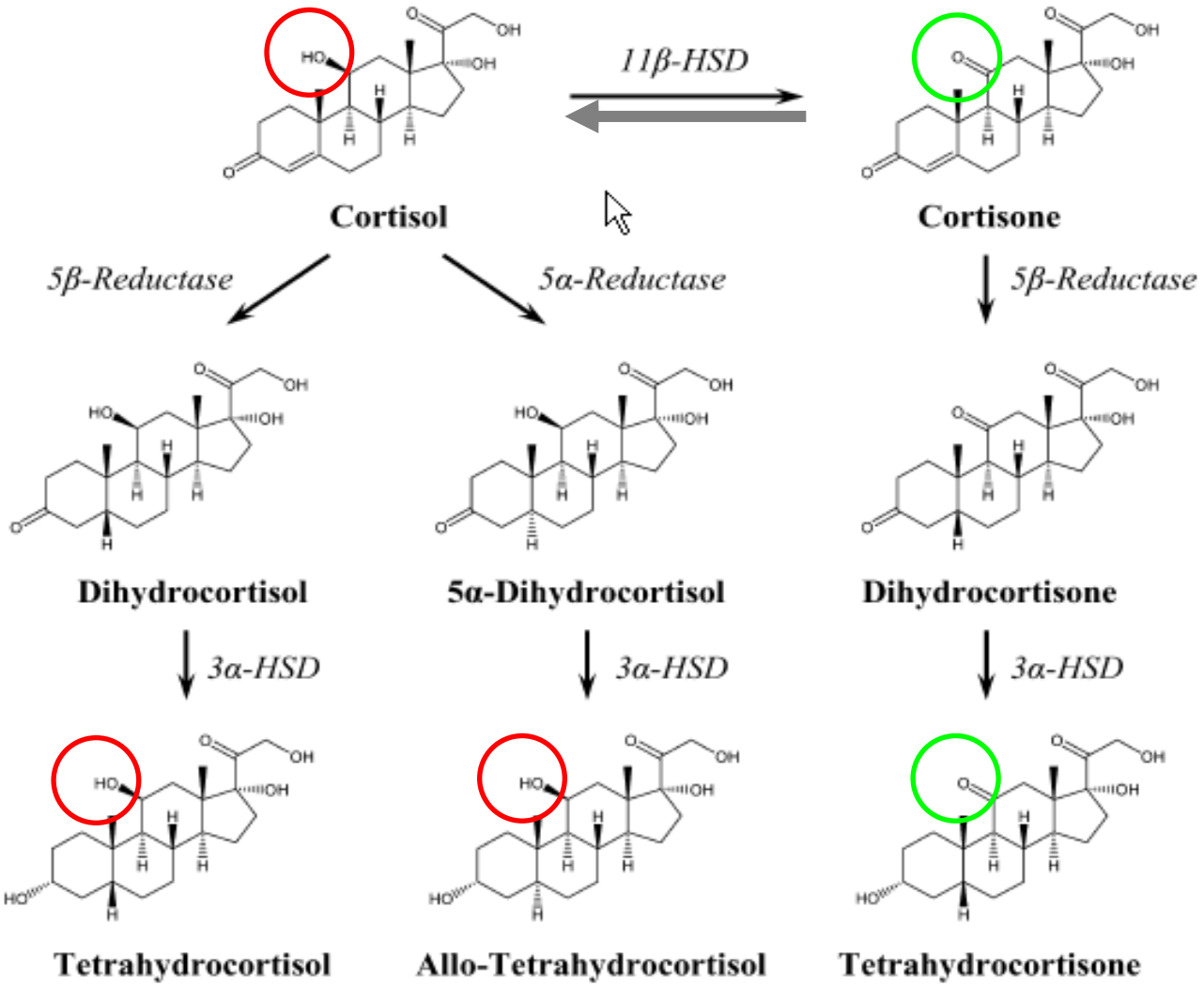
Undiluted Matrix				Diluted Matrix (1:5)				
Matrix	Mean n=6 (ng/mL)	SD	CV	Mean n=6 (ng/mL)	SD	CV	Theoretical	%RE
Control 1	841.7	14.9	1.8	160.3	4.2	2.6	168.3	-4.8
Control 2	610.0	45.5	7.5	120.2	3.0	2.5	122.0	-1.5
Control 3	4993.3	202.5	4.1	954.8	30.7	3.2	998.7	-4.4
Control 4	282.5	17.6	6.2	53.2	1.7	3.2	56.5	-5.9
Control 5	534.5	36.6	6.8	99.9	4.5	4.5	106.9	-6.6
Control 6	2493.3	96.1	3.9	448.5	12.5	2.8	498.7	-10.1

Method Validation – Matrix Effects

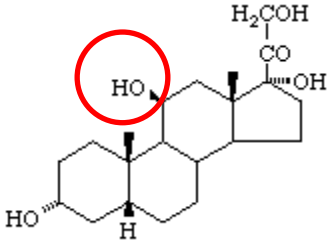
Androsterone in urine

Undiluted Matrix				Diluted Matrix (1:5)				
Matrix	Mean n=6 (ng/mL)	SD	CV	Mean n=6 (ng/mL)	SD	CV	Theoretical (ng/mL)	%RE
Control 1	3481.7	107.0	3.1	1160.0	42.0	3.6	696.3	66.6
Control 2	5435.0	196.0	3.6	1338.3	30.6	2.3	1087.0	23.1
Control 3	544.3	28.2	5.2	117.0	3.2	2.7	108.9	7.5
Control 4	591.7	19.1	3.2	146.0	7.1	4.8	118.3	23.4
Control 5	1153.3	41.8	3.6	249.7	7.7	3.1	230.7	8.2
Control 6	892.7	21.6	2.4	242.3	9.6	4.0	178.5	35.7

Application of Surrogate Matrix Approach - Cortisol Metabolism

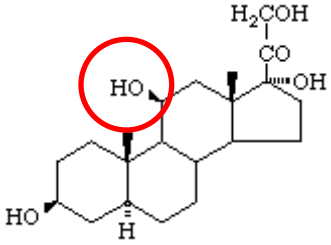


Steroid Ratio 1



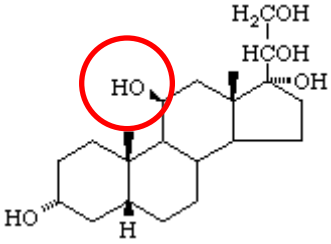
(THF)

+



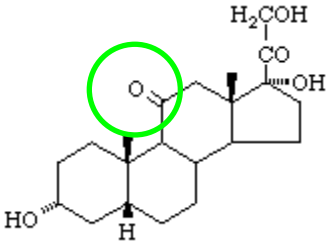
(5α -THF)

+



α- & β-cortol

Steroid Ratio =



THE

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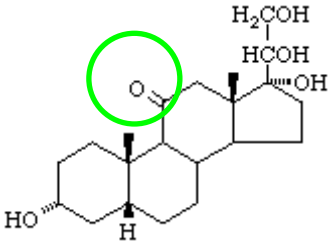


α- & β-cortolone

Indicative of 11ß-HSD1 enzyme inhibition ↓

QC Data from Sample Analysis

QC High	5a-THF	a-cortol	a-cortolone	b-cortol	b-cortolone	THE	THF	Ratio 1	Ratio 2
ng/mL	4540	1150	5210	1250	4890	15800	4070	0.43	0.54
Mean	4558	1215	5273	1320	5005	16831	4055	0.41	0.50
SD	368	81	476	102	441	1309	316	0.02	0.02
%CV	8.1	6.7	9.0	7.7	8.8	7.8	7.8	3.7	4.5
%RE	0.4	5.6	1.2	5.6	2.3	6.5	-0.4	-4.0	-7.4

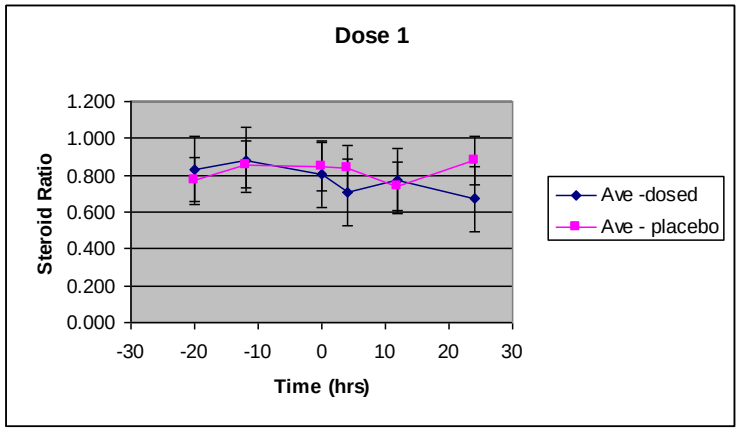
QC Medium	5a-THF	a-cortol	a-cortolone	b-cortol	b-cortolone	THE	THF	Ratio 1	Ratio 2
ng/mL	1620	160	1270	265	938	6140	2180	0.51	0.62
Mean	1530	155	1206	254	913	6072	2131	0.49	0.60
SD	149	13	109	25	87	498	196	0.02	0.03
%CV	9.7	8.4	9.0	10.0	9.6	8.2	9.2	5.0	5.7
%RE	-5.5	-2.9	-5.1	-4.0	-2.7	-1.1	-2.3	-2.5	-3.6

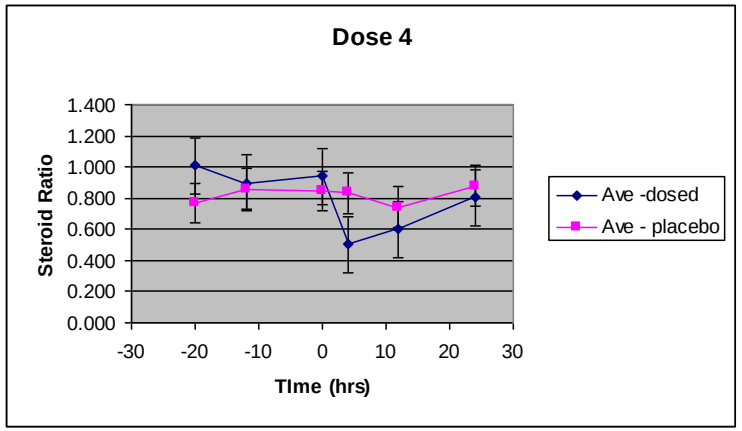
QC Low	5a-THF	a-cortol	a-cortolone	b-cortol	b-cortolone	THE	THF	Ratio 1	Ratio 2
ng/mL	324	32	254	53	188	1230	436	0.505	0.618
Mean	317	31	245	45	188	1141	424	0.51	0.64
SD	41	4	29	7	22	134	54	0.02	0.03
%CV	13.0	12.6	11.8	14.4	11.8	11.7	12.7	3.4	3.9
%RE	-2.2	-3.6	-3.5	-14.6	-0.2	-7.2	-2.8	1.5	4.0

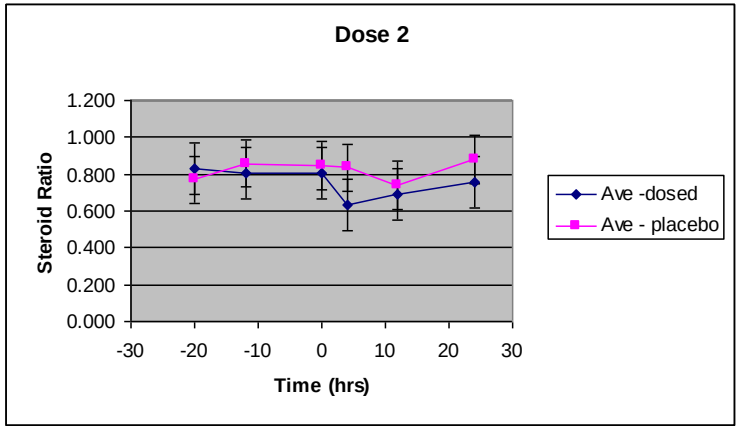
>23% QC failure at +/-15%
<12% QC failure at +/-20%

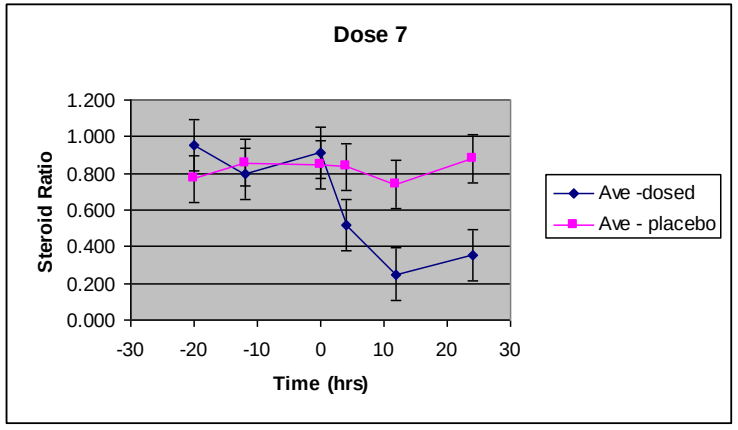
<0.5% QC failure @ +/-15%

SAD Data with Confidence Limits









Generic Approach

METHODOLOGY

Generic approach to validation of small molecule LC–MS/MS biomarker assays

Background: Whilst the regulatory guidelines that describe the validation requirements for small molecules are very comprehensive, they are written primarily for xenobiotic drug molecules. However, the presence of endogenous analyte in control matrix presents an added analytical challenge that must be overcome if small-molecule biomarker assays are to be developed and characterized, especially where downregulation of analyte concentrations is expected. **Experimental:** A generic surrogate matrix calibration protocol has been successfully applied to the measurement of a number of small-molecule exploratory biomarkers using LC–MS/MS. The use of analyte-free matrix enables conventional calibration curves to be constructed across the anticipated range of sample concentrations. The evaluation of matrix effects is carried out using an experiment similar to the parallelism experiment used in ligand-binding assays. **Conclusion:** There is currently no published consensus approach to validation of small-molecule biomarker methods. This paper presents a generic approach to endogenous method validation for consideration as bioanalytical best practice for this type of assay.

Over recent years, there has been a large increase in the use of both exploratory and valid biomarkers [1] as they have been formally incorporated into the drug-development process as indicators of the pharmacodynamic effect of a drug treatment. Whilst there is clear and comprehensive guidance on the expected approach and quality requirements for the generation of regulated pharmacokinetic or bioequivalence data [2,3], this is not the case for biomarker data. Some way has gone to addressing this with the recent paper by Lee *et al.* [4]. Members of the American Association of Pharmaceutical Scientists (AAPS) Ligand-Binding Assay Bioanalytical Focus Group Biomarker Subcommittee collaborated to develop this approach to the validation of laboratory biomarker assays in support of drug development. By their own admission, the focus of the paper was on the use of ligand binding assays for the measurement of biomarker concentrations in *ex vivo* body fluids and tissues. However, biomarkers come in all shapes and sizes and many are small molecules that lend themselves to the use of LC–MS. The traditional expectations for small-molecule assays, in terms of performance, are significantly different to ligand-binding assays for the generation of pharmacokinetic data [5]. However, the validation of a method for the measurement of a biomarker which is an endogenous substance is significantly more complex than the exogenous case and challenges the conventional approach to, and expectations of, even small-molecule assays. This paper presents a generic approach to the validation of small molecule endogenous analyte assays and also extends the discussion initiated by Lee *et al.* on the expectations for small-molecule biomarker assays.

Endogenous assay-specific issues

The majority of small-molecule bioanalysis, performed today, uses LC–MS, but the technique is frequently subject to matrix effects inherent in the ionization mechanism. This is overcome where possible by matrix matching the calibration standards to the unknown samples and the incorporation of stable isotope internal standards into the method to improve assay precision and accuracy. The small-molecule validation guidance documents [2,3], whilst referring to endogenous analytes, are written more specifically for xenobiotics. The presence of the analyte in control matrix presents an extra challenge especially when evaluating the validation parameters of selectivity, recovery, matrix effect and limit of detection. The approach taken in constructing the calibration curve is key to the success of this type of assay. If fortunate, it may be possible to select a control matrix that has a low concentration of endogenous analyte, such as from a different sex or disease state, in which case a conventional matrix calibration is applicable. Another approach demonstrated by Jemal *et al.*

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BIOMARKER
A substance that is measured as an indicator of normal biologic process, pathogenic process, or pharmacologic response to a therapeutic intervention

ENDOGENOUS ANALYTES
A naturally occurring substance found in the sample matrix which is being measured

FUTURE SCIENCE fsg

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- Surrogate matrix calibration
- Generic Approach
- Use of SIL IS
- Applied to both up/down regulation
- Track record of use
- Fit-for-purpose validation
- Applied to wide range of small molecule endogenous analytes

Summary

- Bioanalytical classification of biomarkers; exploratory versus study endpoint
- Use risk based approach to determine extent of qualification/validation
- Opportunity to introduce biomarkers earlier (preclinical) at less cost
- Minimum for a qualification should probably include - Calibration, P&A, Selectivity, Matrix effect, Stability (limited) and Sensitivity
- Consider setting acceptance criteria based on performance of the assay during validation
- Use of surrogate matrix offers a relatively simple generic approach

Quotient Bioresearch

Answers Through Innovation