BIOANALYSIS OF METABOLIC BIOMARKERS

Bioanalysis of Metabolic Biomarkers during Drug Discovery and Early Preclinical Development – Challenges and Solutions

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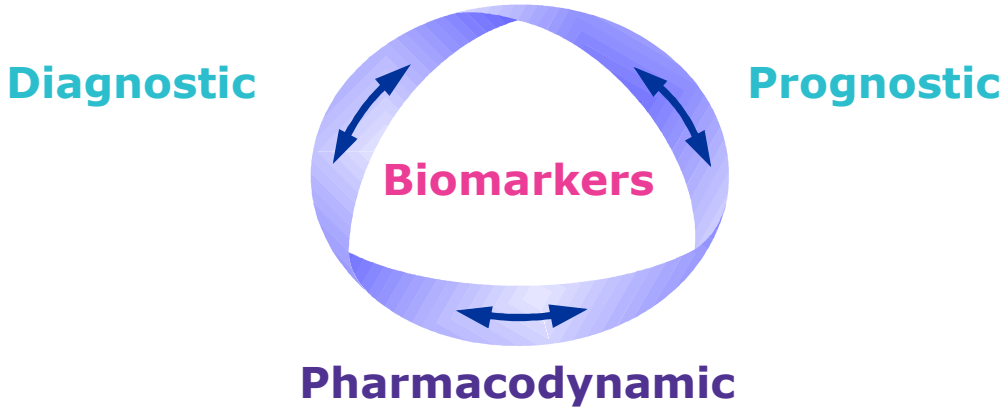
MERCK

Introduction

Metabolic Biomarkers in Drug Discovery and Early Preclinical Development



Biomarkers

- Hallmarks in the context of physiological, metabolic, time and condition related processes
- Targeted, mechanistic insights in drug action, efficacy and safety
- Not static and permanently subjected to change
- Endogenous

Introduction

Fit-for-Purpose Validation Strategies for Biomarker Assays [1]

Basic Performance Parameters

- Accuracy (individual endogenous background needs to be considered if spike recovery of incurred samples is used)
- Precision
- Selectivity (hard to assess for real samples - endogenous background)
- Sensitivity (LLOQ - hard to assess for real samples - endogenous background)
- Linearity (calibration model normally defined in the surrogate matrix)

- Stability
  - Freeze/thaw, bench top, and storage stability based on incurred samples (e.g. from PK/PD Studies)

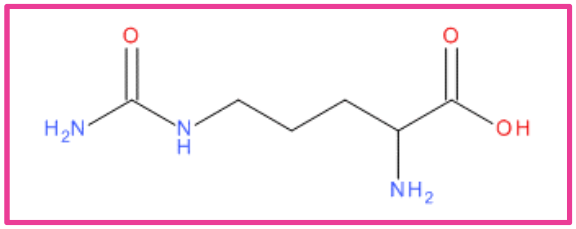
[1] *Lee JW et al.* Pharmaceutical Research **23(2)**, 312-328 (2006).

Introduction

Citrulline – A Potential Translational Safety Biomarker for the Small Intestine

Introduction: Citrulline as a Biomarker

- **Mucosal toxicity** is extremely common following cytotoxic chemotherapy and radiotherapy in the nonclinical and clinical situation.
- The **identification of gastrointestinal mucosal damage at an early stage** with high sensitivity and specifity remains difficult.
- One of potential **noninvasive biomarkers** for intestinal mucosal function and integrity is **plasma citrulline** [2,3].
- Citrulline is an intermediary **metabolic amino acid produced mainly by enterocytes** of the small intestine. A low concentration of free circulating citrulline signifies severe intestinal mucosal damage in humans.
- At present, citrulline is one of the most promising biomarkers used in a clinical setting to quantify the **enterocyte integrity in various small intestinal diseases**.
- Presented case studies investigated the value of plasma citrulline as a translational safety biomarker for unmonitorable intestinal toxicity in **beagle dogs and rats** treated with a reversible and selective peptidase inhibitor, MS-229.



[2] Review: *Crenn P et al.* Clinical Nutrition **27(3)**, 328-339 (2008).
[3] *John-Baptiste A et al.* Toxicologic Pathology **40**, 482-490 (2012).

Case Studies

Bioanalytical Method for Citrulline - „Just Use Water"

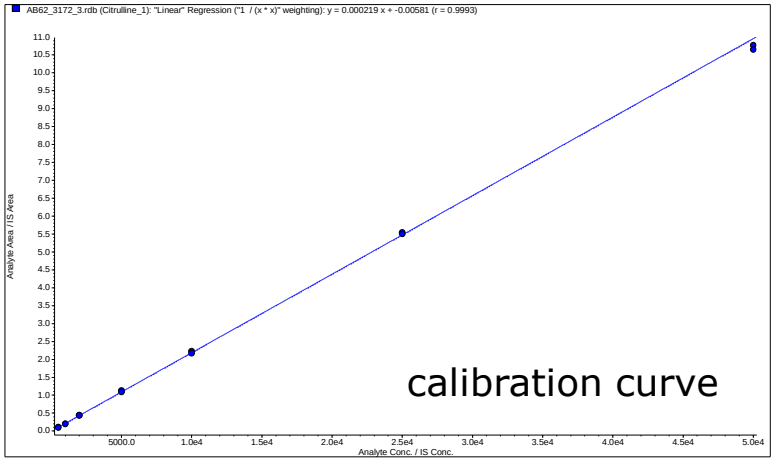
Method Parameters

- IS: Citrulline-D7
- Calibration range 500 – 50.000 ng/mL
- HILIC - ZIC®-HILIC, 50-2.1 mm, 3.5µm
- Eluent A: Formic acid 0.1% (v/v)
  Eluent B: Acetonitrile
- MS/MS conditions:

	Q1 (amu)	Q3 (amu)
Citrulline	176	69.7
Citrulline-D7	183	76.9

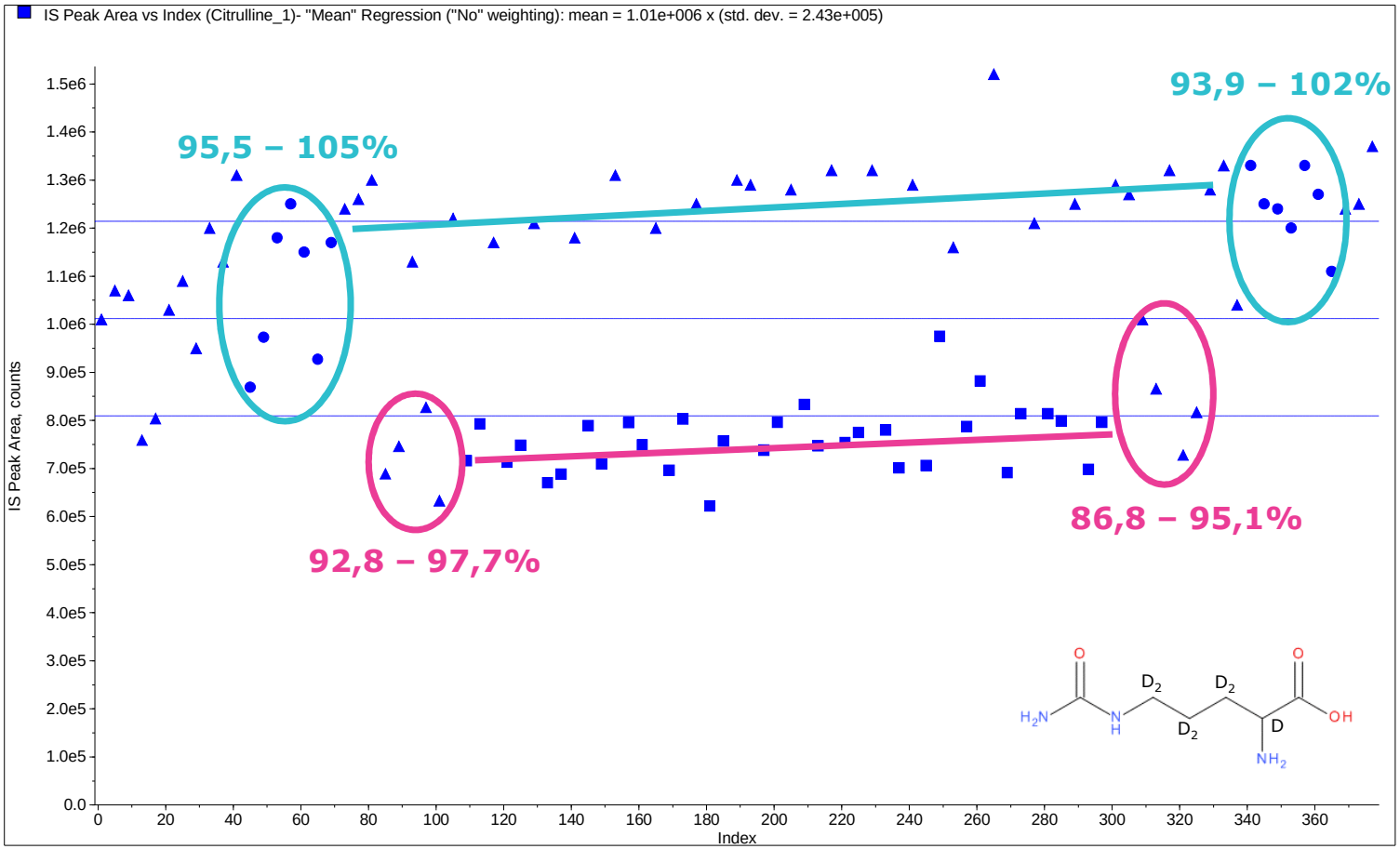
- Sample volume: 5µL
- Injection volume: 5µL; DF:100
- Sample dilution solvent:
  Acetonitrile/Water 90/10 (v/v)

Time (min)	Flow rate (µL/min)	A (%)	B (%)
0.00	400	30.0	70.0
3.00	400	30.0	70.0
3.10	600	30.0	70.0
4.10	600	80.0	20.0
5.10	600	30.0	70.0
6.00	600	30.0	70.0
6.50	400	30.0	70.0
6.60	400	30.0	70.0



Case Studies

Metric Plot of the IS Response in a Citrulline Dog Study Batch



Calibrators & Water Blanks

Unknowns & „Incurred QCs“

Case Studies
Method Performance and Incurred Stability Testing - Rat

Species (matrix: plasma)	sample	First analysis (date)	Second analysis (date)	Third Analysis (date)	First analysis [ng/mL]	Second analysis [ng/mL]	Third analysis [ng/mL]	mean value of first and second analysis [ng/mL]	deviation of second value to the first [%]	mean value of first and third analysis [ng/mL]	deviation of third value to the first [%]
rat	Gr1_m41_d8_3h	20. Nov 13	25. Nov 13	8. Apr 14	15000	13600	16400	14300	**-9,79**	15700	**8,92**
rat	Gr1_f48_d8_3h	20. Nov 13	25. Nov 13	8. Apr 14	15600	15300	17800	15450	**-1,94**	16700	**13,2**
rat	Gr2_m42_d8_6h	20. Nov 13	25. Nov 13	8. Apr 14	9240	8950	8690	9095	**-3,19**	8965	**-6,13**
rat	Gr2_f49_d8_6h	20. Nov 13	25. Nov 13	8. Apr 14	6640	6290	6640	6465	**-5,41**	6640	**0,000**
rat	Gr3_m44_d8_6h	20. Nov 13	25. Nov 13	8. Apr 14	8450	5780	5750	7115	**-37,5**	7100	**-38,0**
rat	Gr3_f51_d8_6h	20. Nov 13	25. Nov 13	8. Apr 14	3440	3170	3420	3305	**-8,17**	3430	**-0,583**
rat	Gr4_m46_d8_6h	20. Nov 13	25. Nov 13	8. Apr 14	4140	4120	4250	4130	**-0,484**	4195	**2,62**
rat	Gr4_f53_d8_6h	20. Nov 13	25. Nov 13	8. Apr 14	4060	3700	3630	3880	**-9,28**	3845	**-11,2**
rat	Gr1_m41_d14_3h	19. Nov 13	25. Nov 13	8. Apr 14	20600	17900	23200	19250	**-14,0**	21900	**11,9**
rat	Gr1_f48_d14_3h	19. Nov 13	25. Nov 13	8. Apr 14	17400	17200	19800	17300	**-1,16**	18600	**12,9**
rat	Gr2_m42_d14_6h	19. Nov 13	25. Nov 13	8. Apr 14	19100	17800	21400	18450	**-7,05**	20250	**11,4**
rat	Gr2_f49_d14_6h	19. Nov 13	25. Nov 13	8. Apr 14	17900	17400	18600	17650	**-2,83**	18250	**3,84**
rat	Gr3_m44_d14_6h	19. Nov 13	25. Nov 13	8. Apr 14	13500	13800	11100	13650	**2,20**	12300	**-19,5**
rat	Gr3_f51_d14_6h	19. Nov 13	25. Nov 13	8. Apr 14	12100	13500	13800	12800	**10,9**	12950	**13,1**
rat	Gr4_m46_d14_6h	19. Nov 13	25. Nov 13	8. Apr 14	13800	12100	14000	12950	**-13,1**	13900	**1,44**
rat	Gr4_f53_d14_6h	19. Nov 13	25. Nov 13	8. Apr 14	9490	10400	9010	9945	**9,15**	9250	**-5,19**
rat	Gr1_31m_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	16600	15600	17000	16100	**-6,21**	16800	**2,38**
rat	Gr1_36f_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	19500	17000	18300	18250	**-13,7**	18900	**-6,35**
rat	Gr2_32m_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	14000	15100	13600	14550	**7,56**	13800	**-2,90**
rat	Gr2_37f_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	15500	15000	15600	15250	**-3,28**	15550	**0,643**
rat	Gr3_34m_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	12100	13100	11600	12600	**7,94**	11850	**-4,22**
rat	Gr3_39f_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	17200	17100	15800	17150	**-0,583**	16500	**-8,48**
rat	Gr4_61m_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	14100	13800	13000	13950	**-2,15**	13550	**-8,12**
rat	Gr4_64f_d14_2h	21. Nov 13	25. Nov 13	8. Apr 14	16800	16400	16600	16600	**-2,41**	16700	**-1,20**
		days:	**4-6**	**138-140**				Mean:	**-4,36**	Mean:	**-1,23**

- Method performance and reproducibility on 3 consecutive days
- Incurred sample reproducibility + 1x freeze/thaw, 1x bench–top
- Incurred sample stability over 138 days minimum (+ 2x F/T, 2x BT)

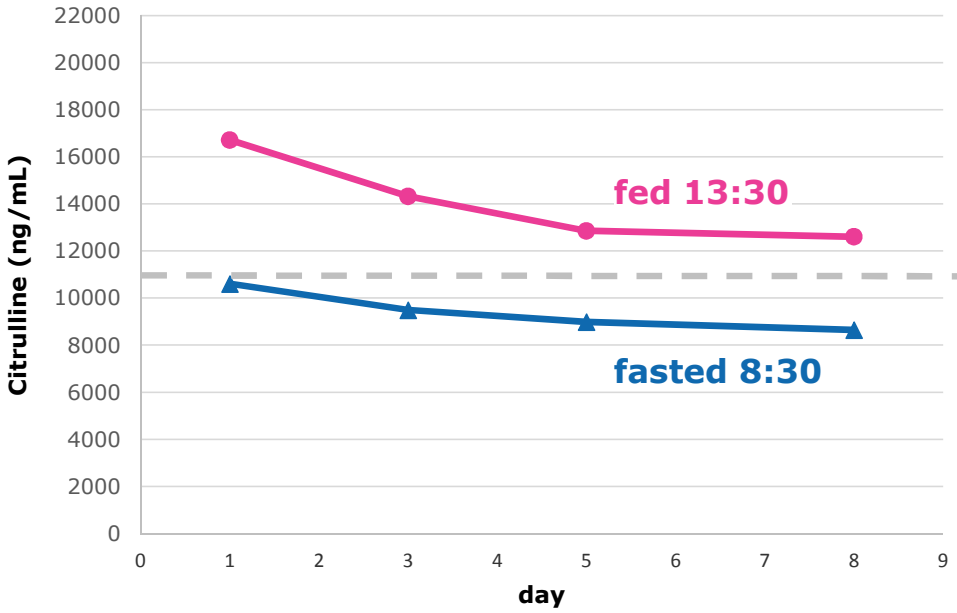
Case Studies
Method Performance on 3 Consecutive Days - Rat

AB62_3249	Citrulline	Calibration Range: 500 - 50000 ng/mL			**Performance Summary**	
date		19. Nov. 13		20. Nov. 13		21. Nov. 13
batch		AB62_3249_2		AB62_3249_3		AB62_3249_4
accepted Ks		**18 out of 18**		**18 out of 18**		**18 out of 18**
r		0,9981		0,9976		0,9995
accepted QCs		**12 out of 12**		**12 out of 12**		**12 out of 12**
QCs spike	ng/mL	20 000	ng/mL	20 000	ng/mL	20 000
	Samples	Accuracy (%)	Samples	Accuracy (%)	Samples	Accuracy (%)
set 1	QC1 013	97,0	QC1 013	95,0	QC1 013	92,2
	QC2 014	95,2	QC2 014	97,8	QC2 014	100
	QC3 015	98,7	QC3 015	104	QC3 015	99,2
	QC4 016	114	QC4 016	109	QC4 016	97,3
	QC5 017	104	QC5 017	104	QC5 017	100
	QC6 018	103	QC6 018	103	QC6 018	98,4
set2	QC1 116	101	QC1 116	97,5	QC1 132	91,7
	QC2 117	101	QC2 117	94,3	QC2 133	92,3
	QC3 118	98,7	QC3 118	98,7	QC3 134	107
	QC4 119	101	QC4 119	100	QC4 135	101
	QC5 120	85,5	QC5 120	99,0	QC5 136	98,6
	QC6 121	92,8	QC6 121	96,5	QC6 137	99,4
mean value		96,5		97,7		98,4
STD		6,22		2,05		5,82
initial	G2_43m_d14_9h 084	15900	G2_43m_d8_24h 084	9110	G2_33m_d14_4h 071	13400
concentrations	G2_50f_d14_9h 085	16300	G2_50f_d8_24h 085	6820	G2_38f_d14_4h 072	12500
(ng/mL)	G3_45m_d14_9h 098	15300	G3_45m_d8_24h 098	4620	G3_35m_d14_4h 085	12400
	G3_52f_d14_9h 099	15000	G3_52f_d8_24h 099	6670	G3_40f_d14_4h 086	13500
	G4_47m_d14_9h 112	13900	G4_47m_d8_24h 112	6110	G5_63m_d8_4h 128	3870
	G4_54f_d14_9h 113	13600	G4_54f_d8_24h 113	3450	G5_66f_d8_4h 129	2140

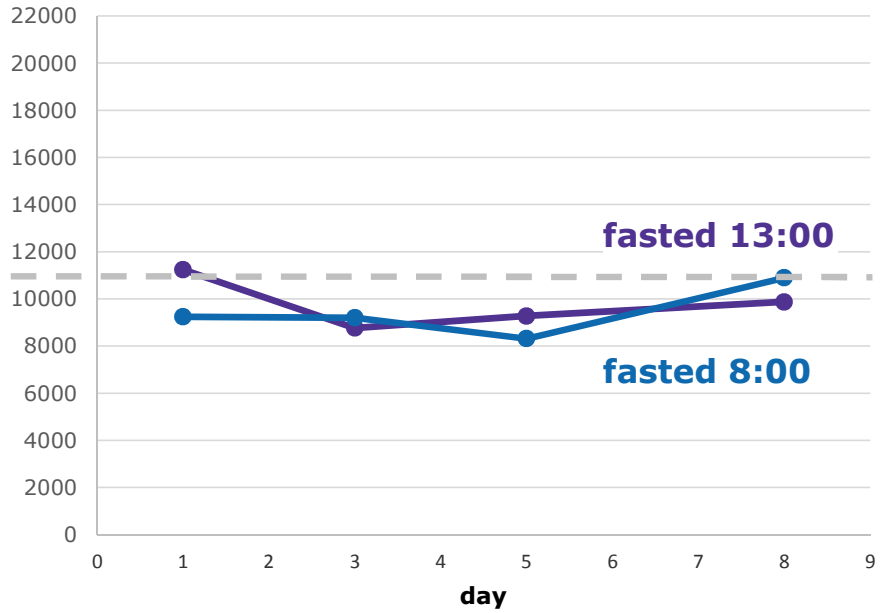
Case Studies

Citrulline Levels in Fasted and Fed State Dogs – Mean Values

Mean citrulline levels in fed vs. fasted dogs



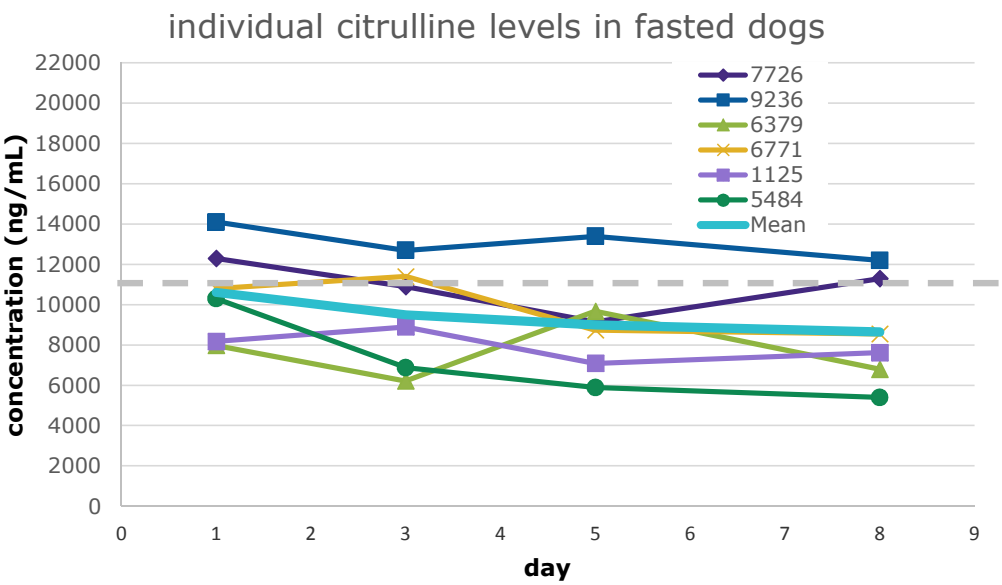
Mean citrulline levels in fasted dogs "circadian features"

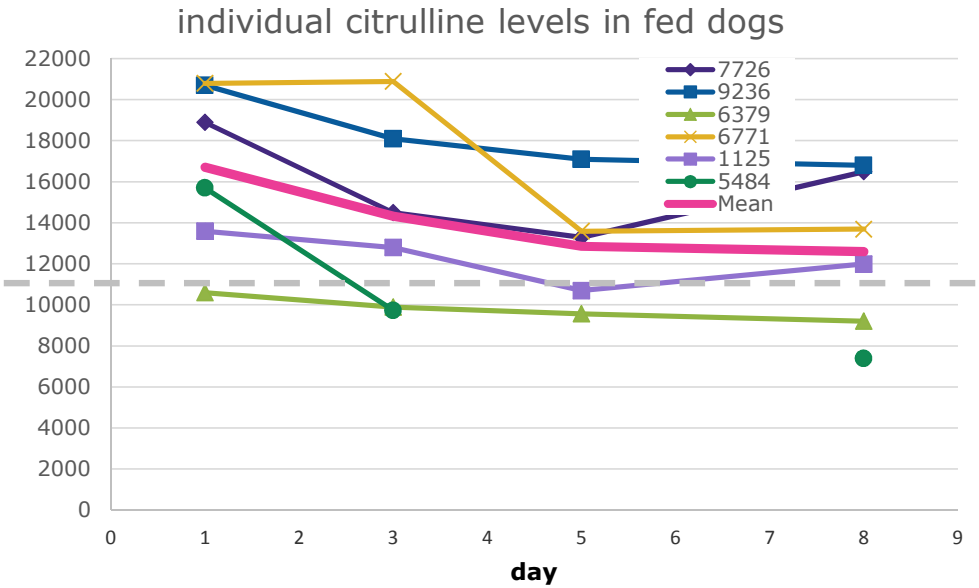


- Citrulline levels in fed dogs higher

Case Studies

Citrulline Levels in Fasted and Fed State Dogs

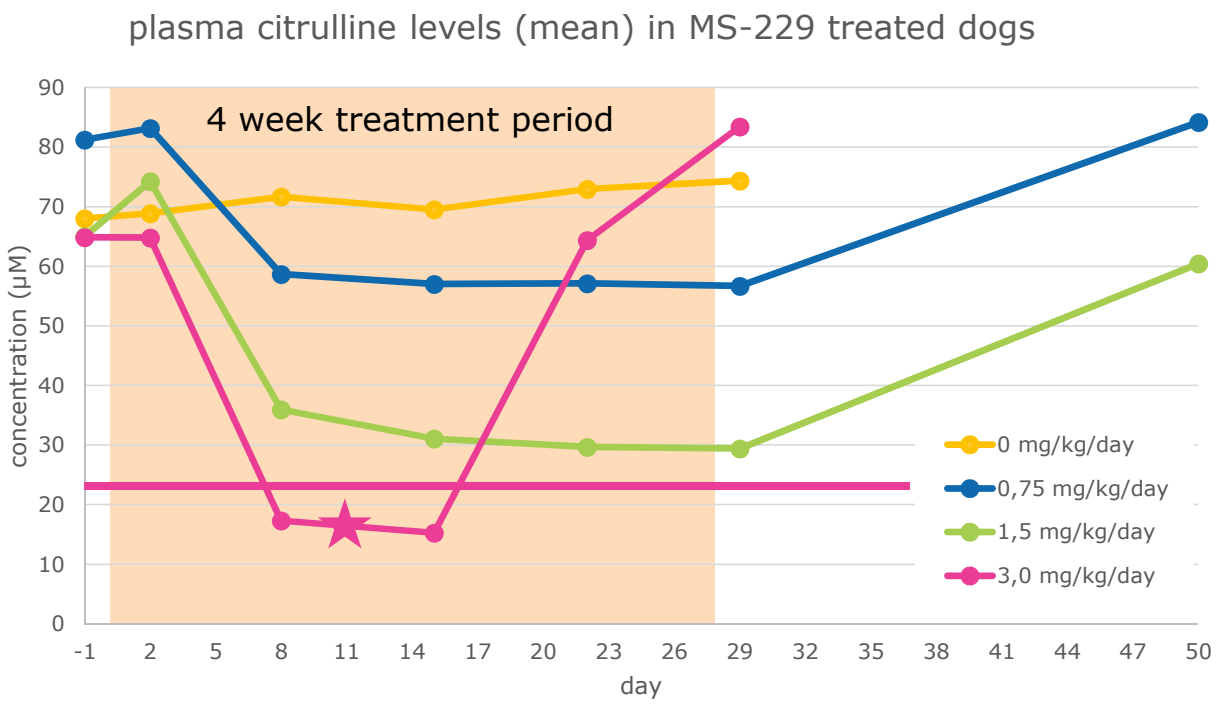




- Citrulline levels in fed dogs higher
- Reasons and significance unclear – high degree of variation from animal to animal

Case Studies

Plasma Citrulline Levels in MS-229 Treated Dogs



★ Treatment of high dose group stopped due to adverse clinical symptoms on day 11

Histopathology Findings:

0.0 **No findings**

0,75 **Clinically tolerated**

No morphological alterations

1,5 **FC and BW moderately decreased**

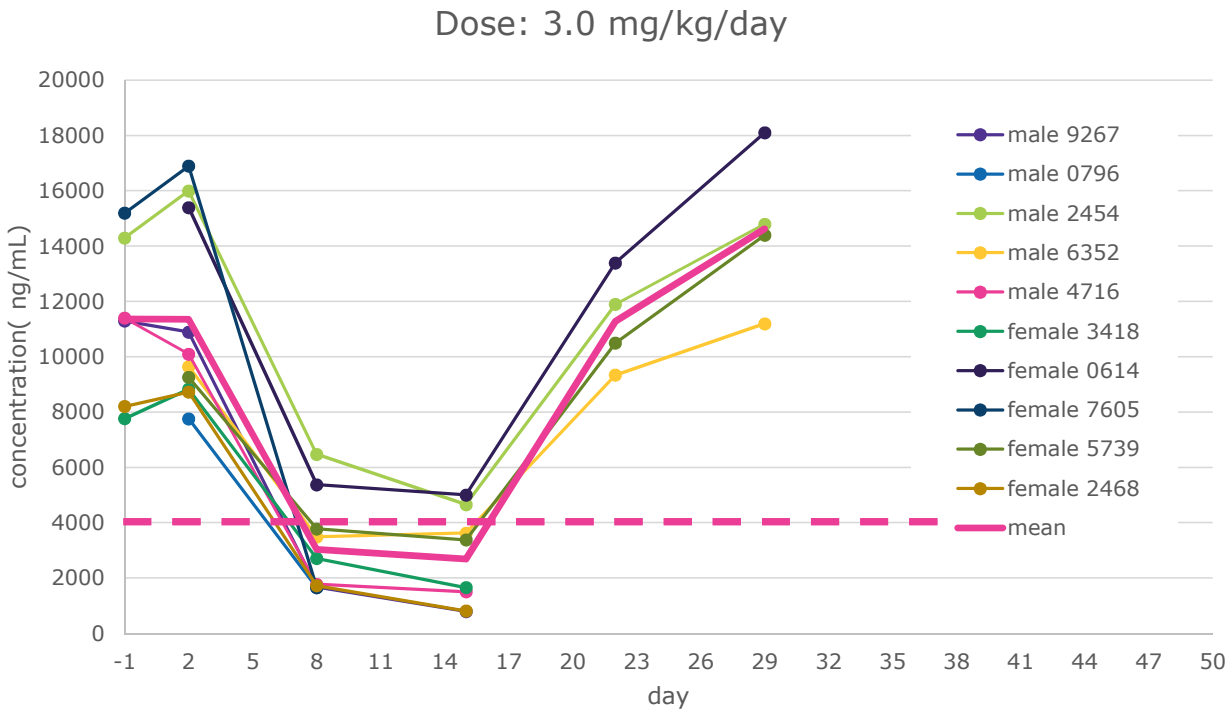
Reversible, mild to moderate mucosa degeneration

3,0 **Clinically not tolerated**

Reversible, moderate to marked mucosa degeneration

Case Studies

Plasma Citrulline Levels in MS-229 Treated Dogs



Biomarker data of individual subjects:

- Variance not liked to analytical performance
- Significance lower due to normal biological variance

- Especially composite profiles (e.g. from rats/mice) are hard to interpret due to higher variations
- → serial microsampling

Conclusions
Summary

Biomarker Method Qualification/Validation

- Tiered Fit-for-Purpose validation strategies are well suited to deal with the unique challenges of biomarker assays.
- Incurred sample reanalysis and incurred stability assessments are a fully acceptable alternative for classical validation routines.
- No issues with the quantification/method validation of citrulline observed so far.

Citrulline – A potential translational safety biomarker for the small intestine

- Dose- and exposure-dependent decrease in plasma citrulline observed in dogs after repeated oral administration of MS-229.
- Decrease of citrulline in dogs correlated very well with pathological findings in small intestine.
- For dogs a cut-off value for intestinal mucosal toxicity was established.
  - approx. 4000 ng/ml (23 µM)
- However, analysis of data from individual animals is essential!

Conclusions
Future Perspectives

Biomarker Method Qualification/Validation

- Continuation of the Citrulline Method Validations based on the current experiences.

Citrulline – A potential translational safety biomarker for the small intestine

- Implementation of plasma citrulline as a small intestine biomarker into dog safety studies needs to be further validated.
- Prerequisite for a mature understanding of biomarker biology are highly resolved experiments (animal numbers / time-points).
  - currently ongoing at Merck
- Cross-species translation: rats/mice/monkey plasma biomarkers correlation to small intestine toxicity needs to be addressed.
  - Initial data from rat: not clear-cut!
- Translation to man?
  - Implementation into clinical studies with MS-229 as an exploratory biomarker planned.

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Thank you for your attention!