Validation of a new ACT*One*™ - FLIPR^{TETRA}® assay for measurement of peptide activity in plasma samples

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Assays for measurement of peptides in plasma

Plasma concentrations of peptides are often very low e.g.

- GLP-1: endogenous 5-40 pM
- Human insulin: endogenous 50-500 pM

- The exogenous concentrations are often higher
- Immuno assays are traditionally used
- Metabolites can be agonists, antagonists and immuno reactive

Immuno assays

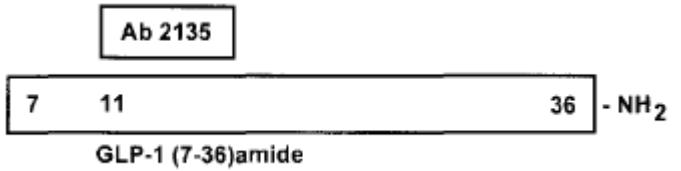
1. RIA/EIA where only one antibody is used

2. "Sandwich" assays where two antibodies bind to the peptide or protein at the same time

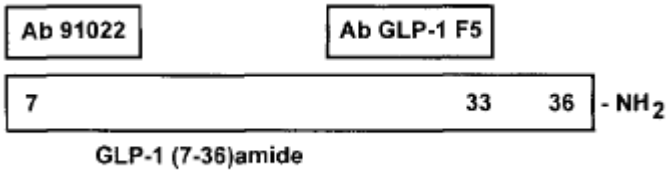
- The antibody recognizes an "epitope" which consists of 10-15 amino acids
- Immuno reactive metabolites are quantified
- Immuno reactivity and not concentration is measured
- It takes > 6 months to make a new antibody

Example: GLP-1 assays

- RIA (antibody with epitope from aa 11 to aa xx)



- Sandwich ELISA (N-terminal specific antibody + antibody with epitope from aa xx to aa 33)



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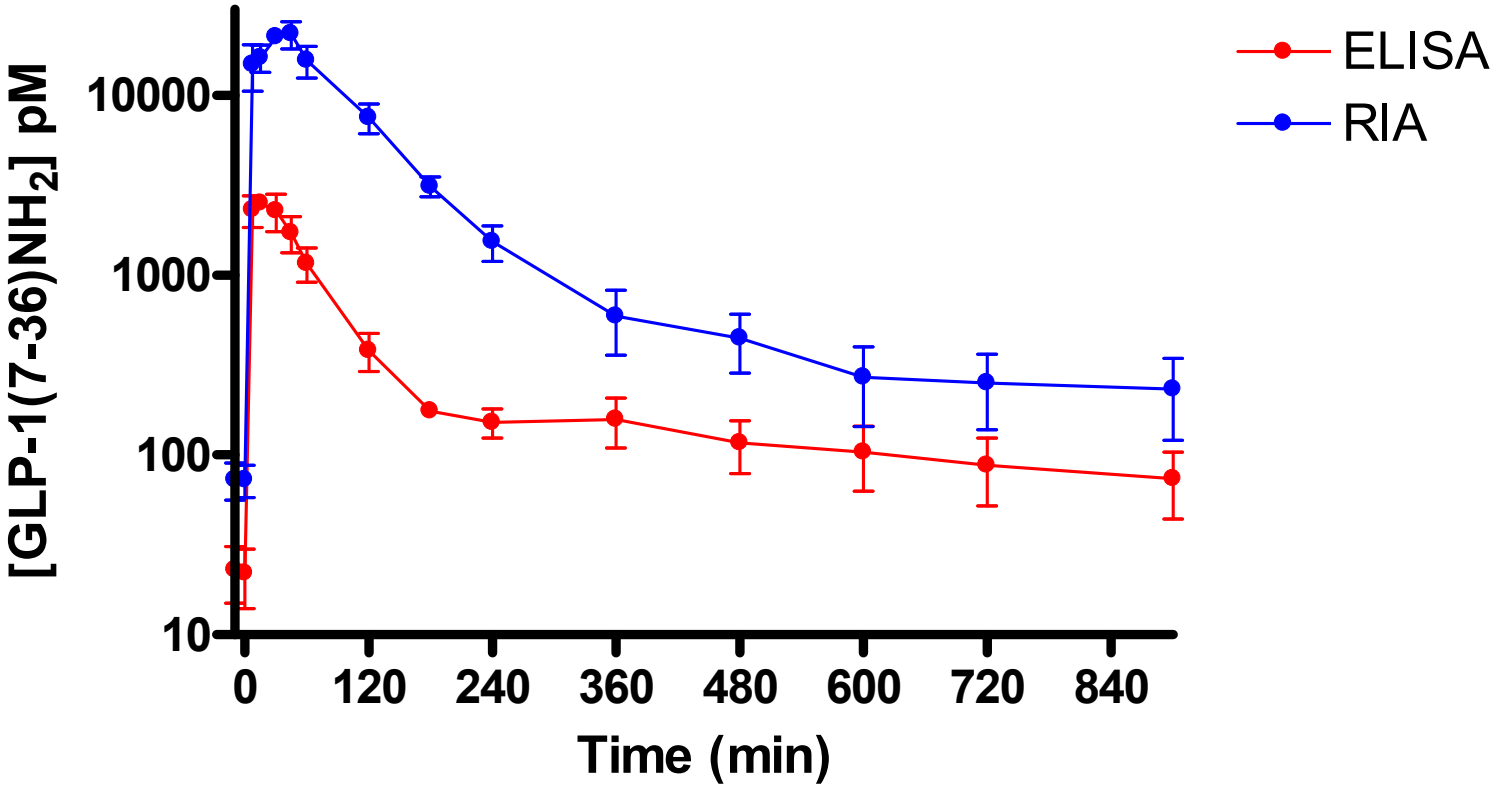
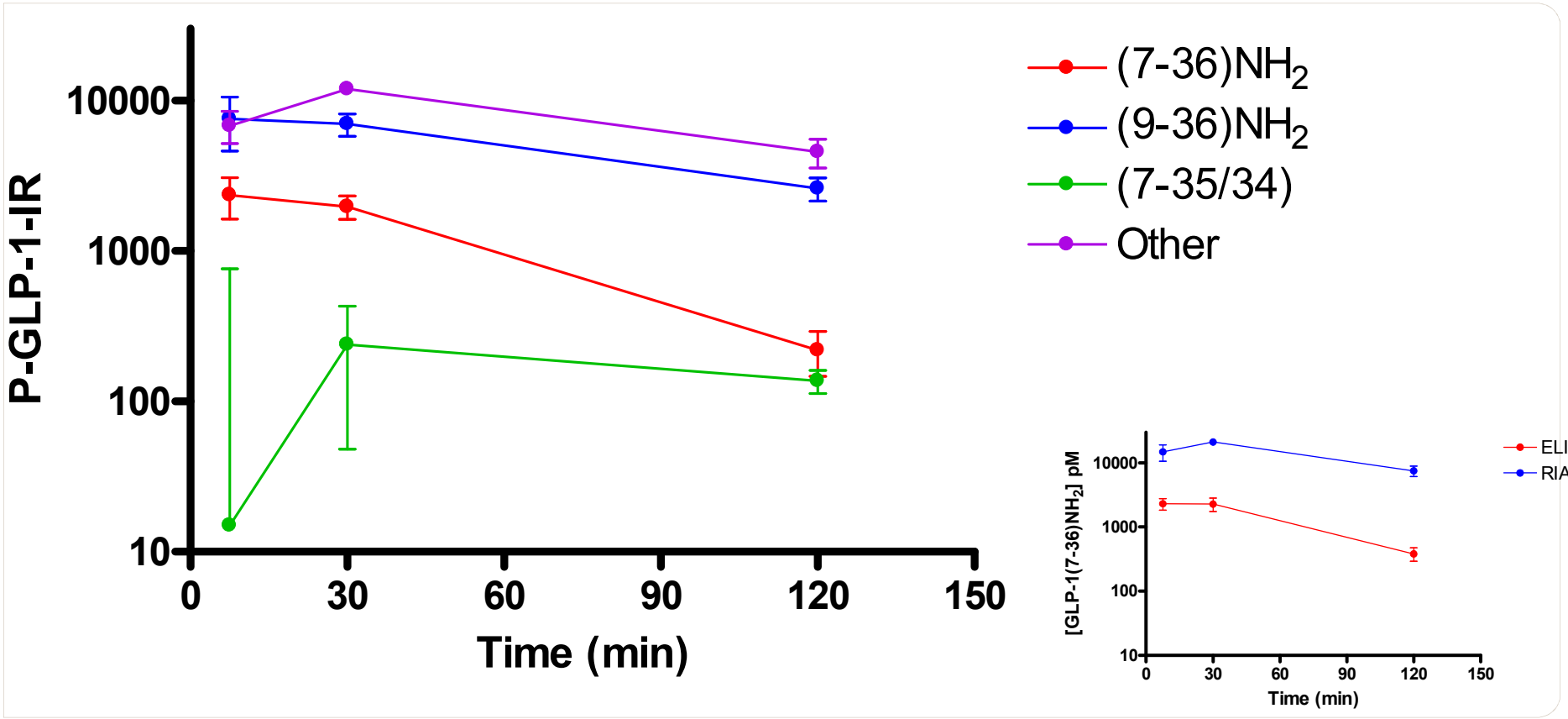
Plasma concentration curves for GLP-1(7-36)amide 15 nmol/kg s.c. to dogs



Metabolites of GLP-1(7-36)amide in dogs after s.c. administration



Other = Metabolites truncated beyond Glu^{9} in the N-terminus and/or beyond Lys^{34} in the C-terminus

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New assay types

- Needed to screen for modified peptides, where no antibody is available
- Early preclinical screening of many modified peptides (no antibodies available)

1. LCMS

2. Receptor based assays

LCMS

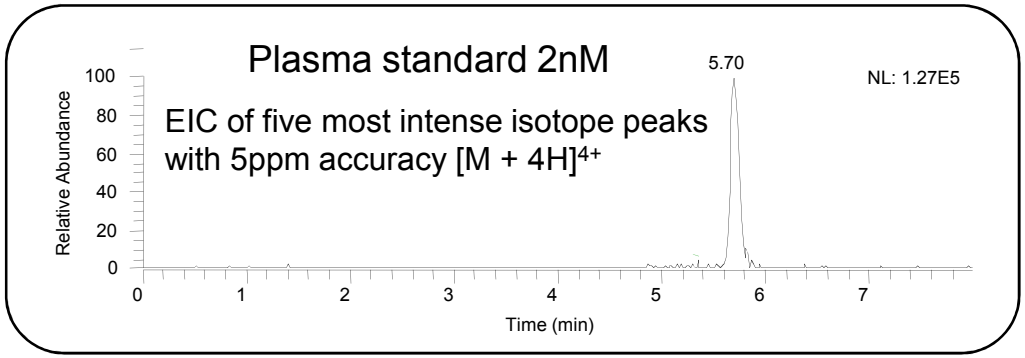
- HPLC
- Detection by MS

➢ Throughput low (240 samples per day)

➢ Sensitivity is usually low, i.e. required detection limit may not be met

➢ The parent compound is quantified (no metabolites included)

LC-MS for Peptide Analysis In Vivo (Plasma)



- Plasma samples are typically precipitated with organic solvent (EtOH/MeOH, Acetonitrile (1% HCOOH)). The recovery is usually >70-80%.
- In other projects, turboflow chromatography or solid phase extractions are used for sample preparation.

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Slide from Dr. Jørgen Olsen, Novo Nordisk A/S

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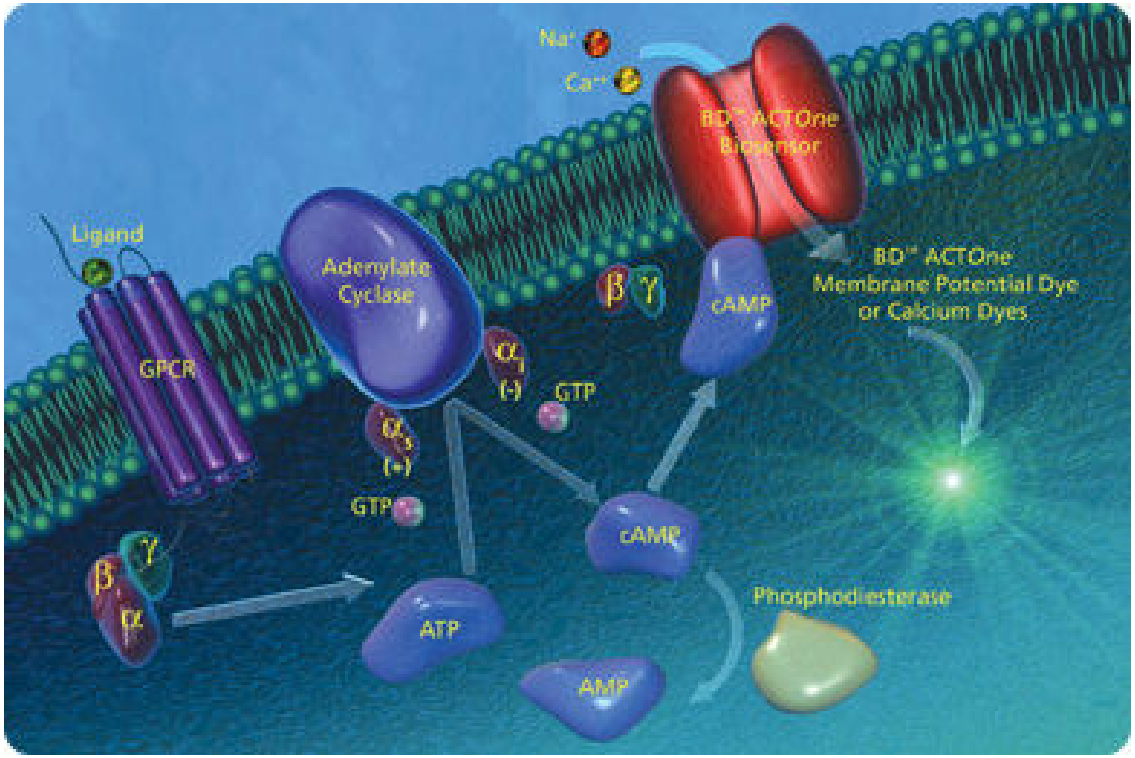
Receptor based assays

- Quantification of parent compound and metabolites that are agonists or antagonists

1. Binding to the receptor
   - Agonists
   - Antagonists (measured as an addition)
2. Activation of the receptor
   - Agonists
   - Antagonists (measured as a subtraction)

New type of GPCR assay - ACT*One*™



BD ACTOne™ cAMP Biosensor
BD ACTOne™ is an easily scaleable cAMP biosensor HTS platform for Gs and Gi GPCR drug discovery and deorphanization.

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Sample preparation

- 96 well filter plates (0.45µ hydrophobic)
- 100 µl 96% Ethanol
- 10 µl plasma
- Mix
- Centrifugation
- Evaporation in the UltraVap™ (Porvair Sciences, UK)
- Dissolution in 60 µl HBSS-buffer containing 20mM Hepes, 0.1% Ovalbumin, 0.005% Tween 20

Cell preparation

- Format: 384
- Cells: 14.000 cells/well in 25 µl
- Over night at 37°C and 5 % CO_2
- Dye: 25 µl FLIPR® Calcium 4 Assay Kit
- 1 h at 37°C followed by 1 h at RT

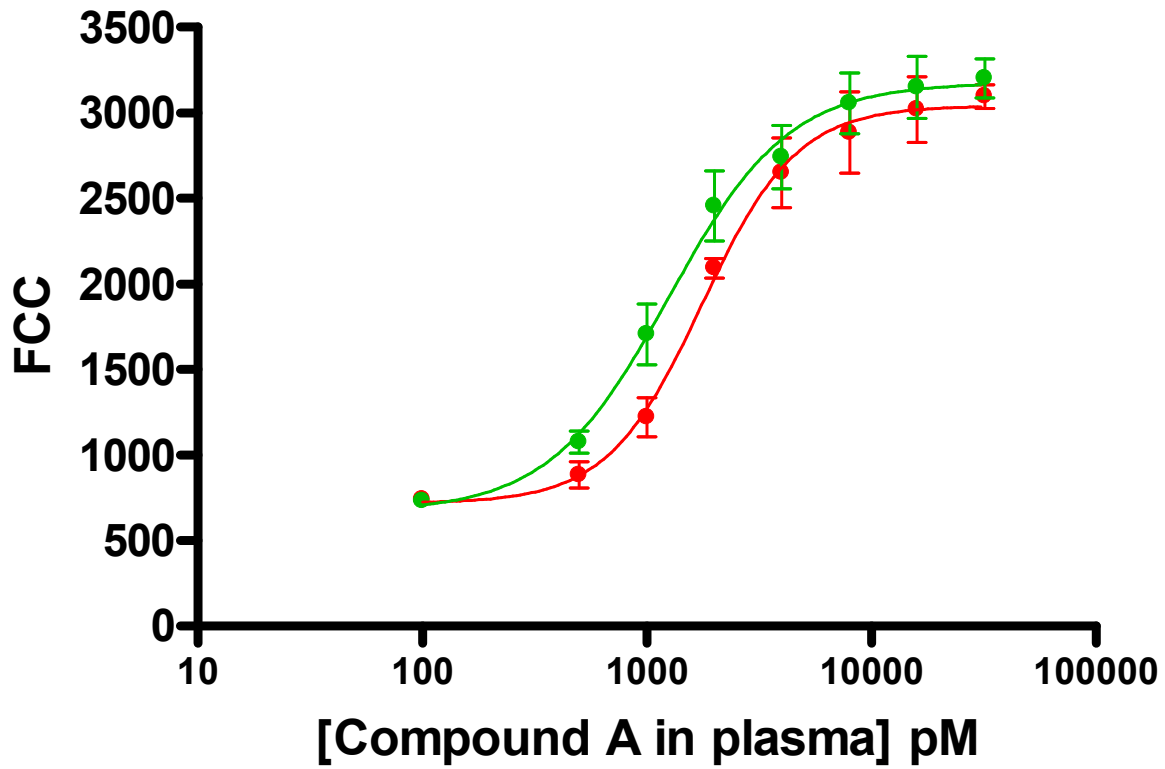
FLIPR protocol

- λ:
  - excitation 470-495 nm
  - emission 515-575 nm
- Timing:
  - inject t= 30 sec
  - measure t= 360 sec
- Volume
  - 5 µl (≈ 0.8 µl plasma or 63 fold dilution of plasma)

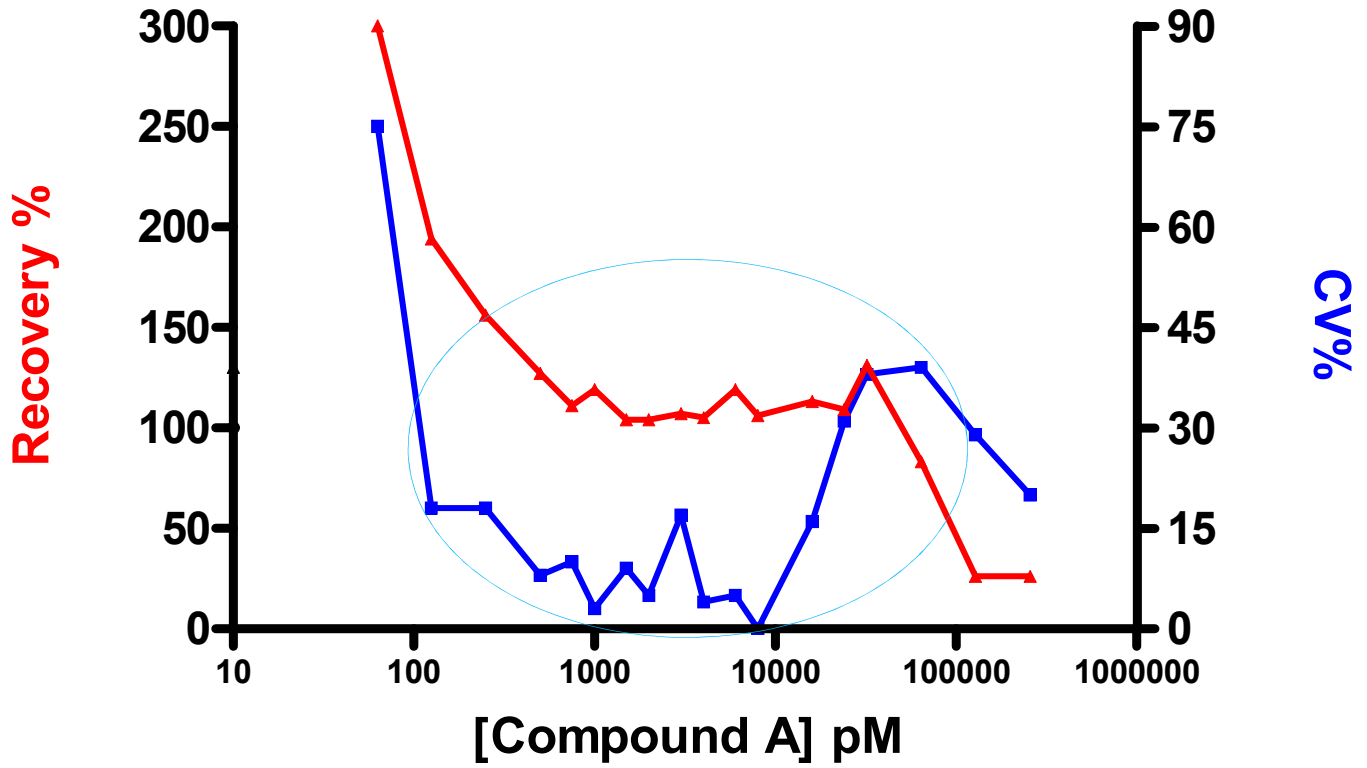
Compound A

- An agonist which binds to a Gs coupled receptor
- Modified peptide
- Highly albumin bound
- Low receptor affinity (100x lower than endogenous agonist)
- No known/available antibodies

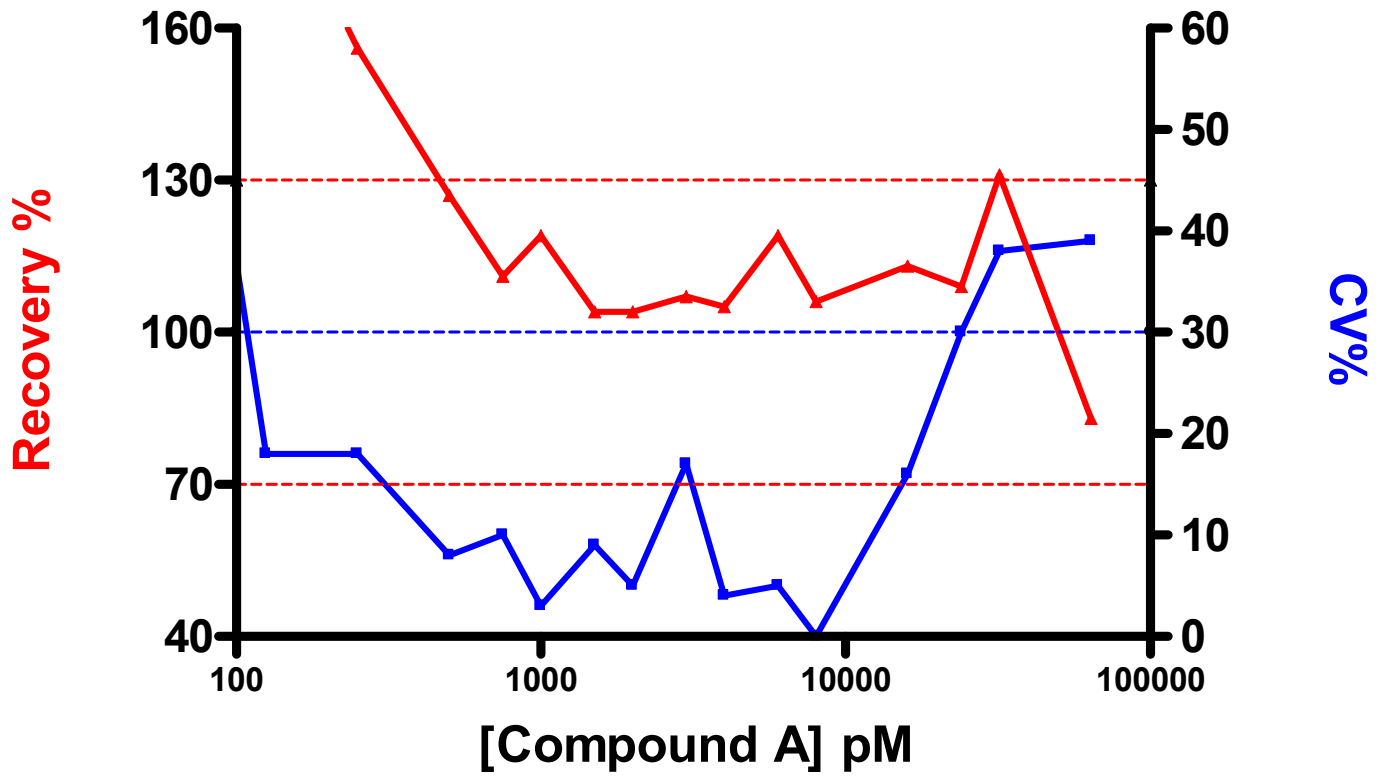
Dependence on species



Determination of analytical range



Determination of analytical range



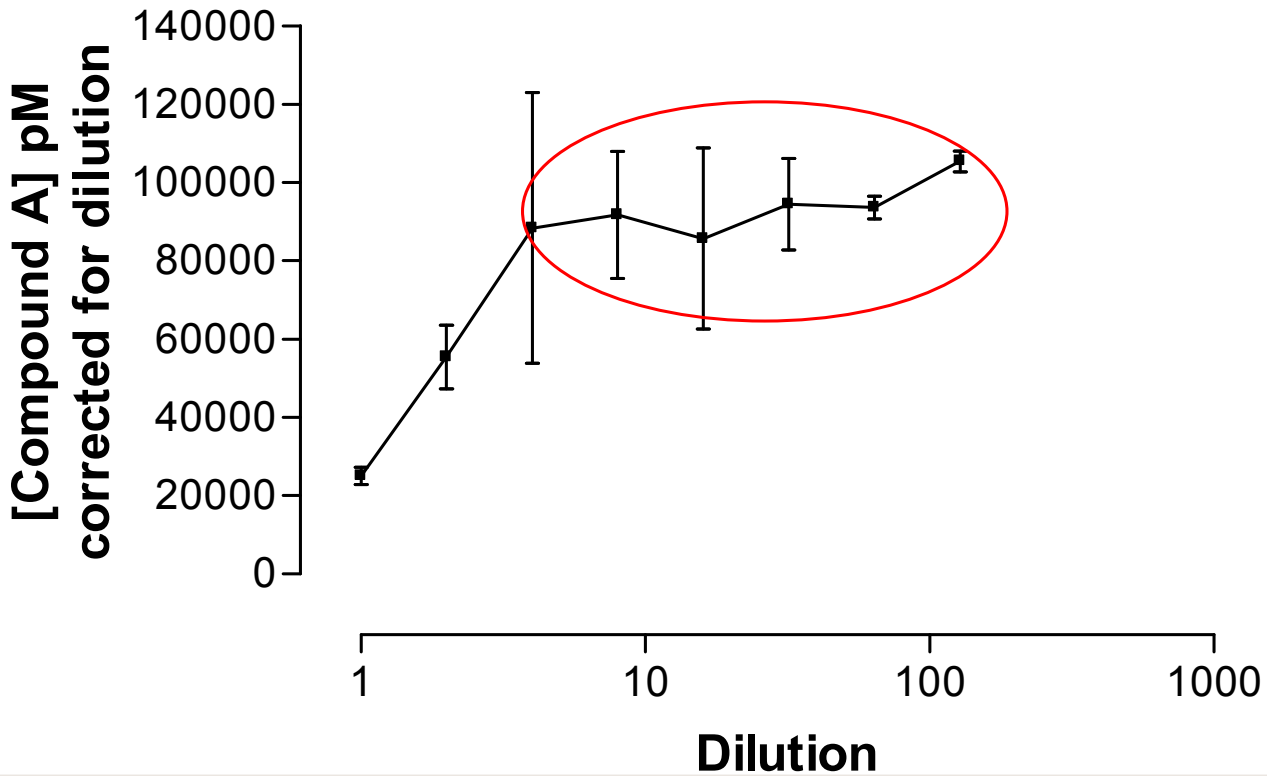
Determination of analytical range

	LOD	LLOQ	HLOQ
1st test	250	500	24000
2nd test	250	500	40000
Result	250	500	20000

Day to day variation – 5 spiked samples

	Average (pM)	SD (pM)	CV (%)	Recovery (%)	Minimun (pM)	Maximun (pM)
25000	30000	8202	27	120	20700	36200
10000	10550	1975	19	106	8550	12500
5000	5553	503	9	111	5110	6100
2500	2725	545	20	109	2110	3260
1500	1503	343	23	100	1190	1450
500	607	57	9	121	575	683

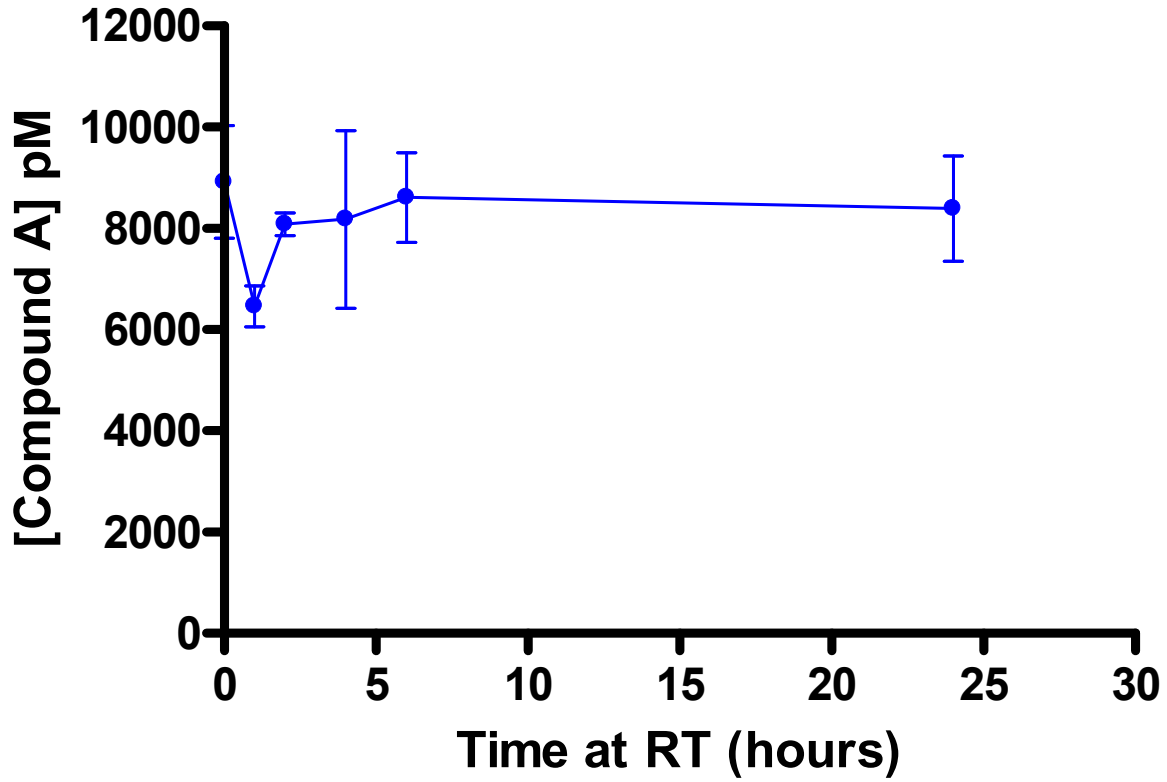
Dilution Recovery



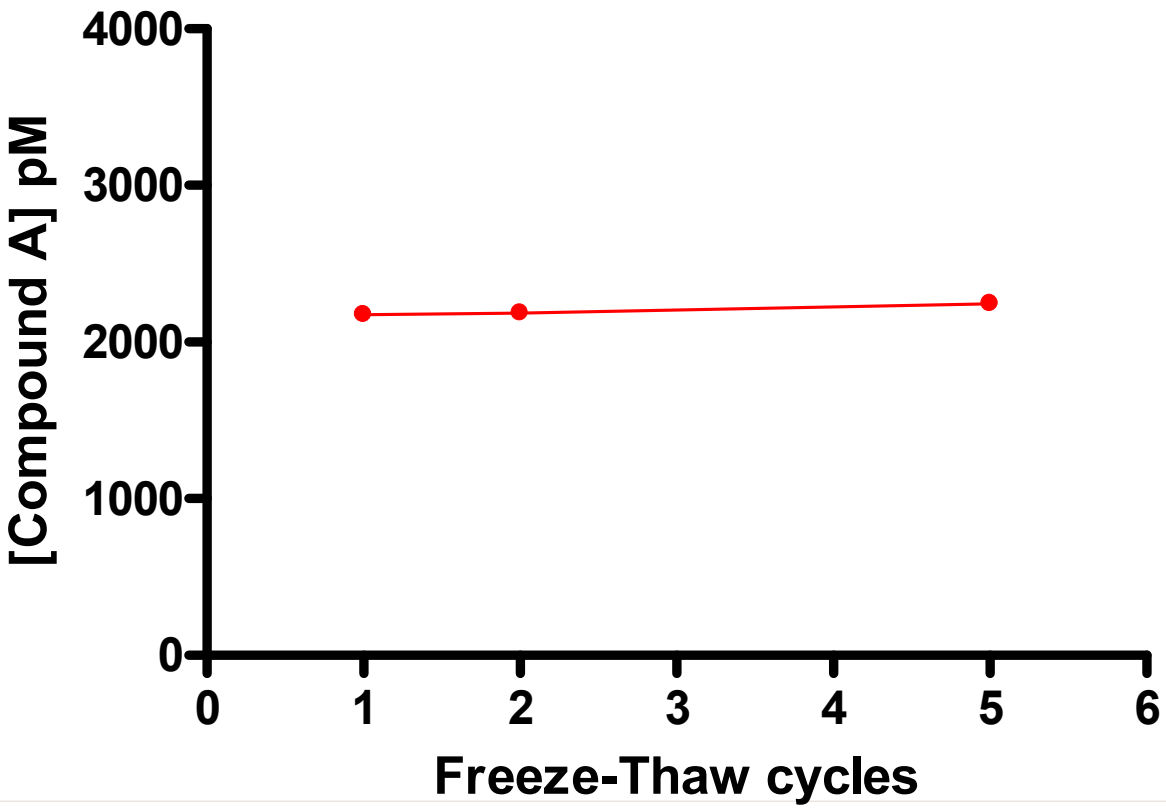
Spiking recovery

	Average (pM)	SD (pM)	CV (%)	Recovery (%)
25000	43825	3876	9	175
12500	14650	1242	8	117
6250	5598	262	5	90
3000	2270	686	30	76
1500	1370	397	29	91
750	610	63	10	81

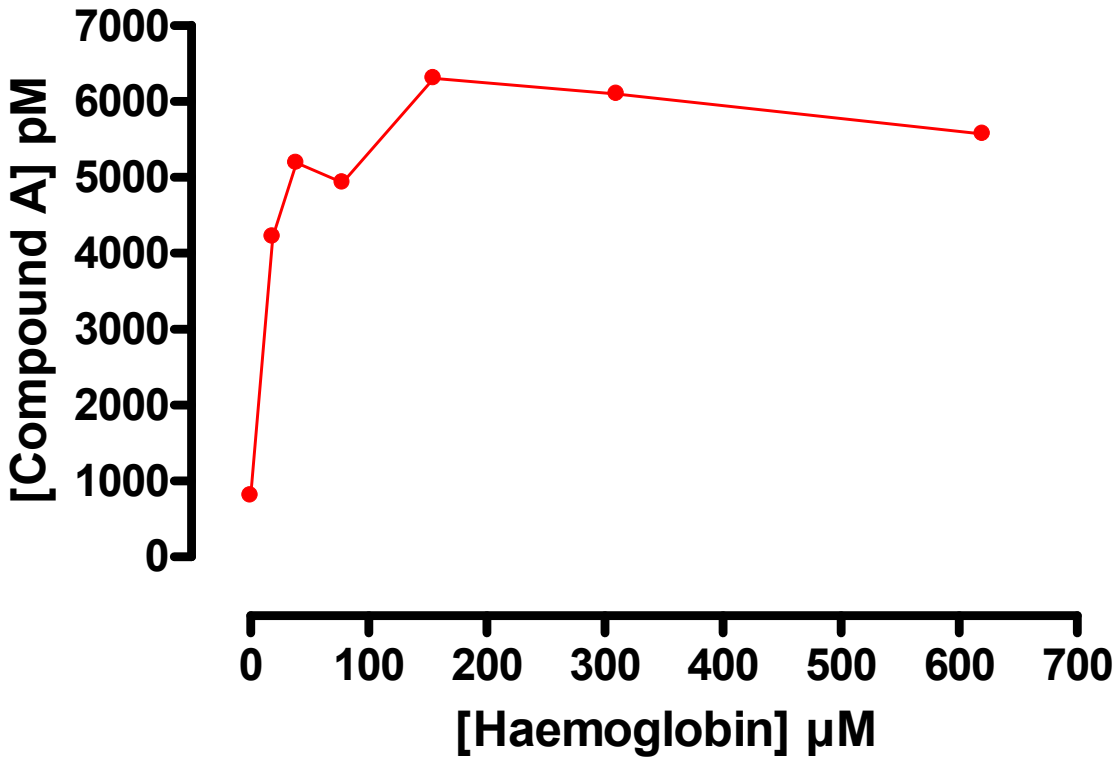
Stability of the plasma samples



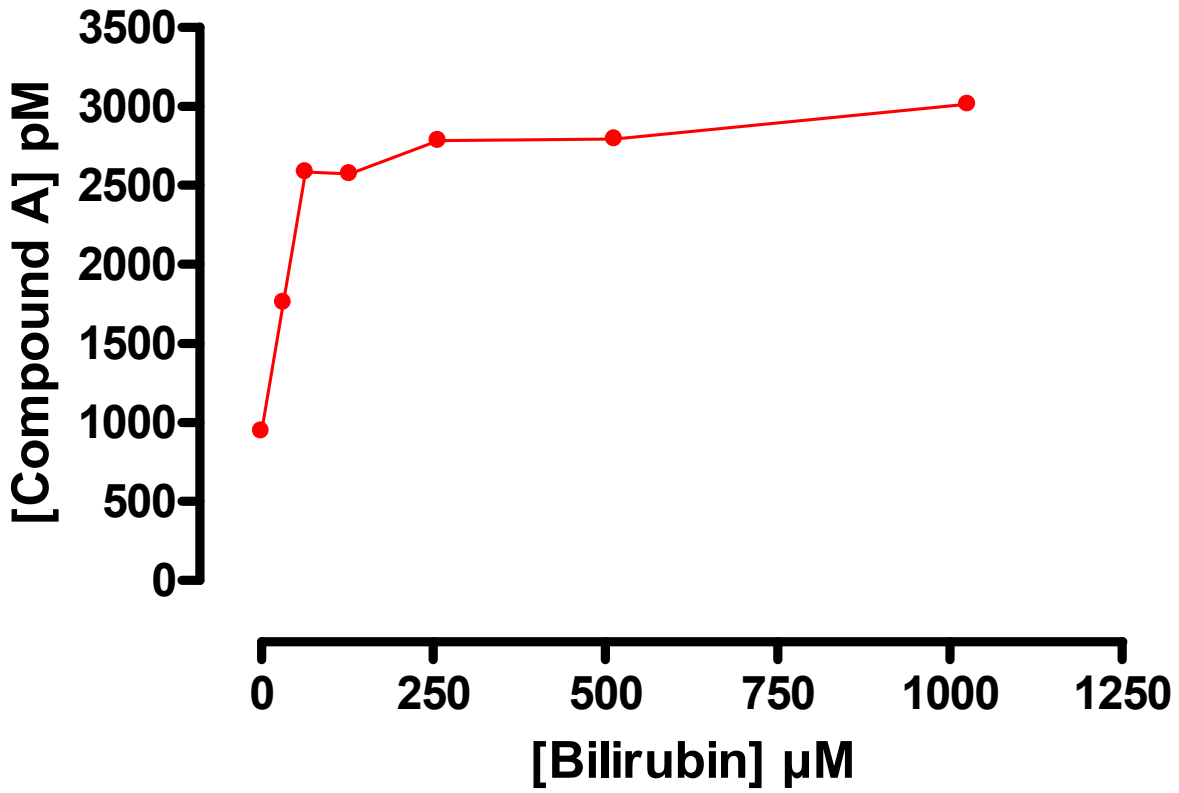
Freeze-Thaw stability



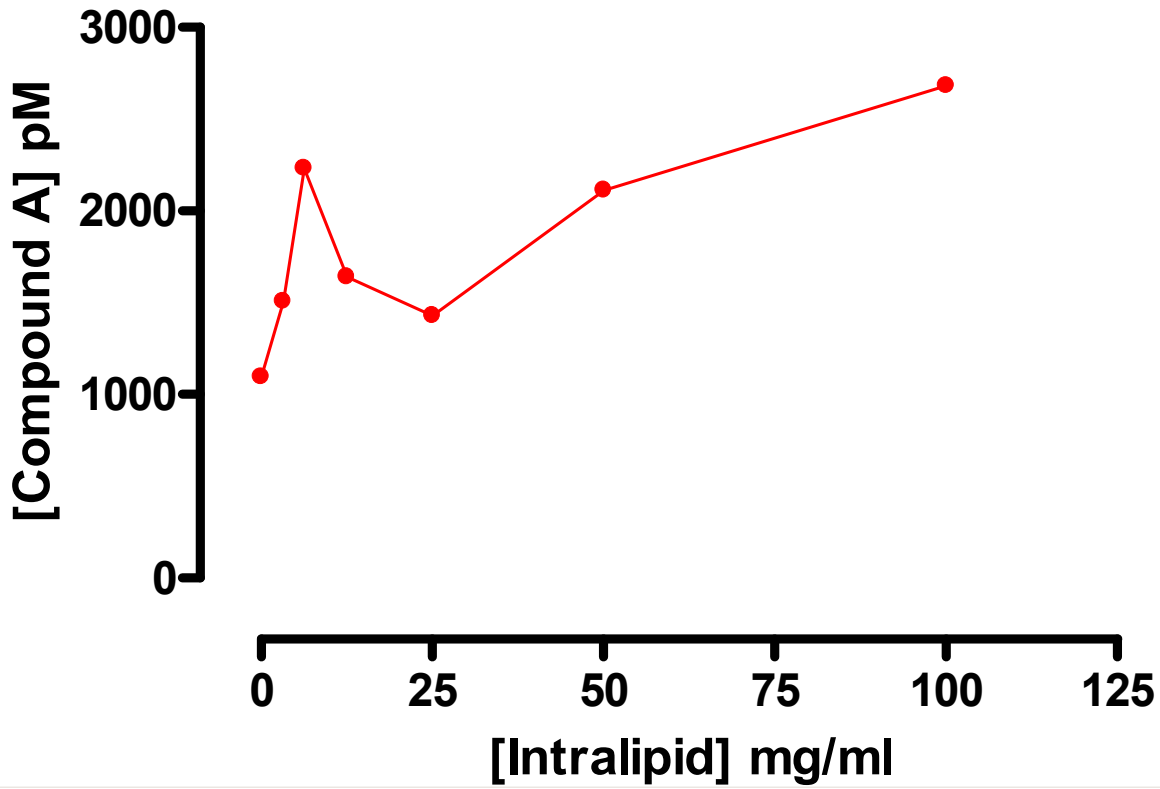
Interference by Haemoglobin



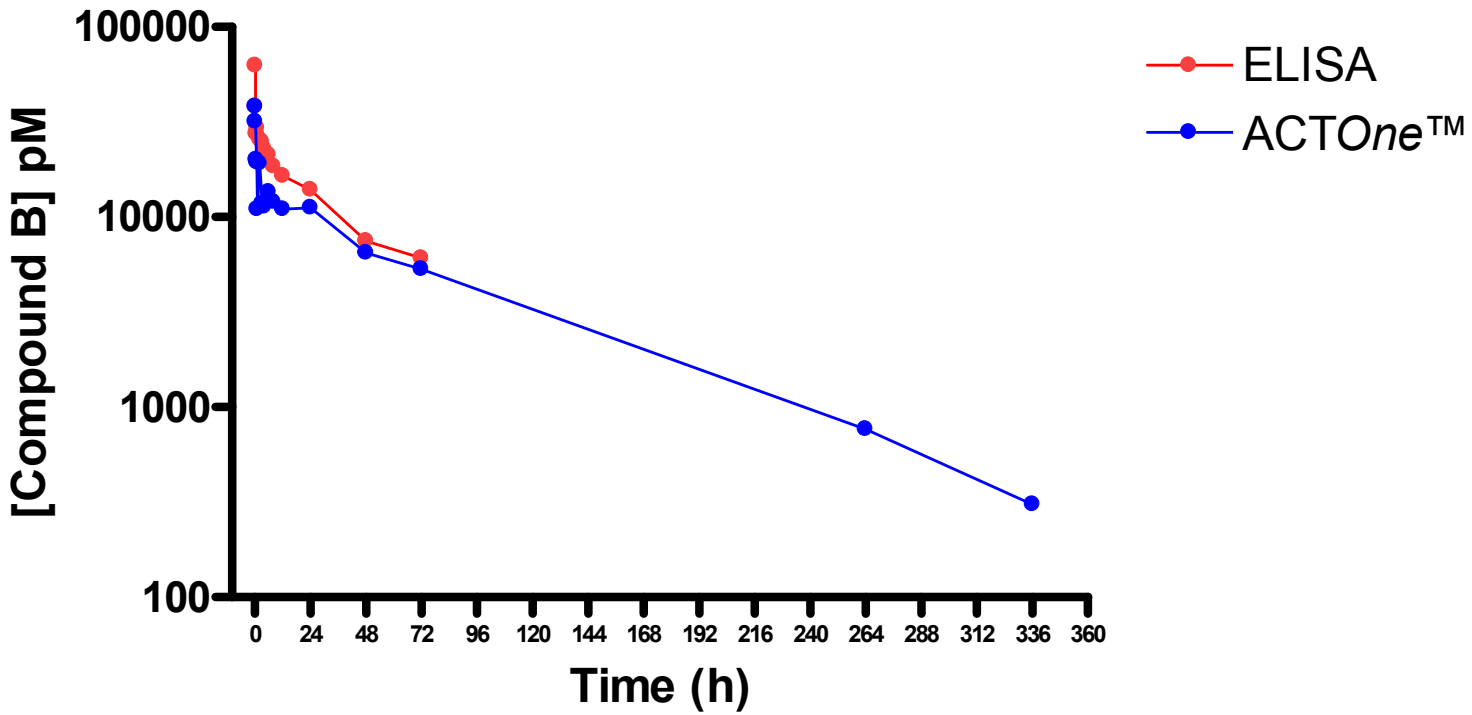
Interference by Bilirubin



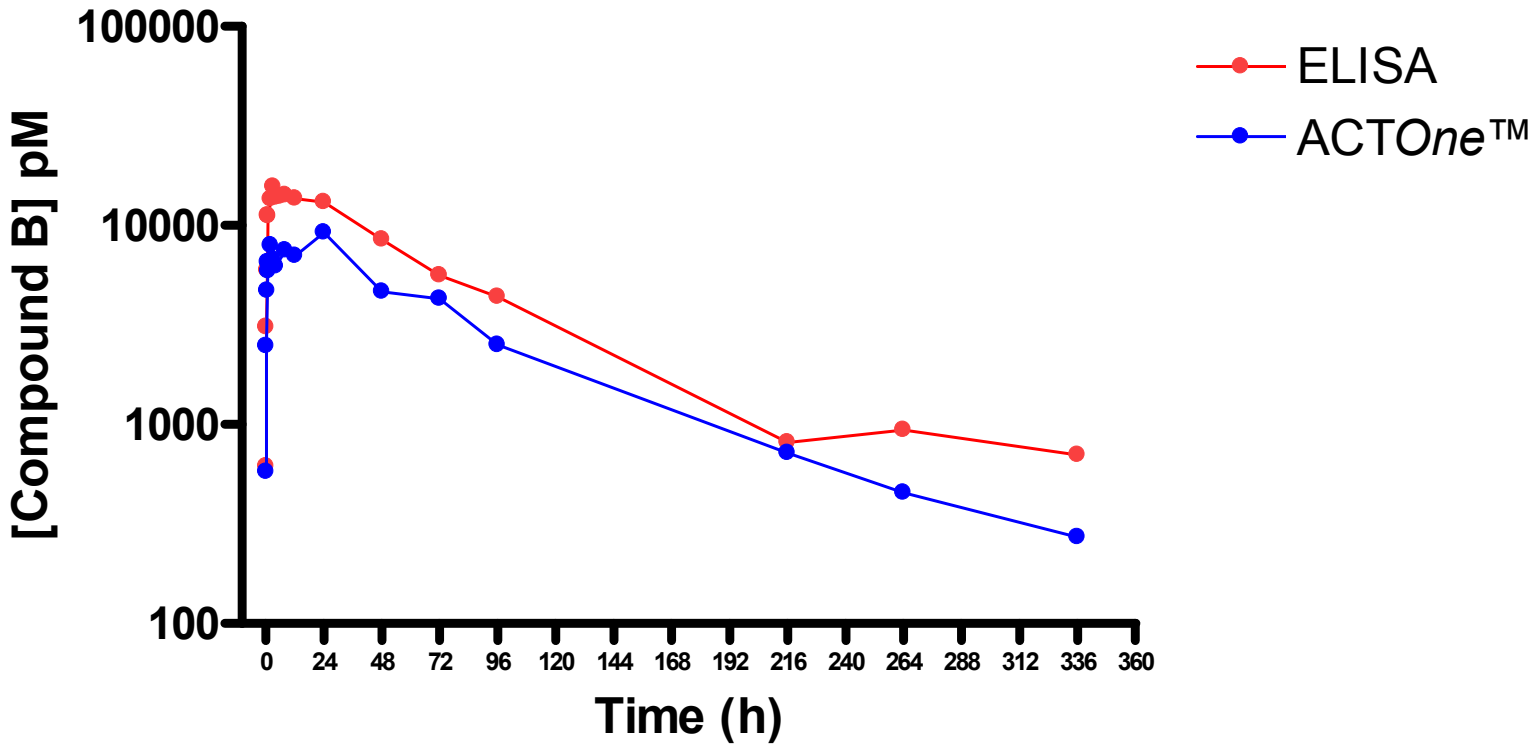
Interference by Intralipid



Comparison with ELISA assay – i.v. dosing



Comparison with ELISA assay – s.c. dosing



Analytical range - conclusion

- Recovery 70-130 %
- CV < 30 %

- Analytical range: 500-20.000 pM

- Samples are routinely tested undiluted and 10x diluted in duplicates (4 wells per sample)

- Analytical range: 500-200.000 pM with one extra dilution

Analytical range - FDA requirements

- Recovery 80-120 %
- CV < 20 %

- Analytical range: 2.000-16.000 pM

- Samples tested undiluted, 5x diluted and 25x diluted in duplicates routinely (6 wells per sample)

- Analytical range: 2.000-400.000 pM with two extra dilutions

Comparison to other assays

ASSAY	RESULT	LLOQ (pM)	Throughput samples/day	Sample Preparation
RIA	Parent compound + Immuno reactive metabolites corrected for cross reactivity	10-100	1000	?
Sandwich IA	Parent compounds + Immuno reactive metabolites corrected for cross reactivity	1-10	8000	no
Receptor binding assay	Parent compound + Metabolites (Agonists + Antagonists corrected for cross reactivity)	100-1000	3000	yes
Receptor activation assay	Parent compound + Metabolites (Agonists – Antagonists corrected for cross reactivity) = activity	50-1000	3000	yes
LCMS	Parent compound	250-25000	240	yes

GLP-1 Assays

At t=120 min:	**[GLP-1(7-36)amide]:**	**219 pM**
	[GLP-1(9-36)amide]:	**2595 pM**
	[GLP-1(7-34/35]:	**137 pM**
	[Other]:	**4548 pM**

ASSAY	MEASSUREMENT		
RIA	219+1.13*2595+1.48*137+4548	=	7902 pM
ELISA	219+0.01*2595+0.90*137	=	368 pM
ACTOne	219-0.01*2595+0.08*137	=	204 pM
LCMS	219	=	219 pM

Conclusion – ACTOne assay

- Sensitive assay (dependent upon potency of parent compound)
- Medium throughput
  - >3.000 samples per day (≈ 42 x 384 well plates)
  - Duplicates
  - Undiluted and 10x diluted
- Activity measurement, not immuno reactivity or concentration
- To be used when no antibodies are available and throughput exceeds LCMS capacity
- Works with Gs and Gq coupled receptors

Acknowledgements

- Annette Hansen
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