

# Validation of a new **ACTOne™ - FLIPR<sup>TETRA</sup>®** 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.

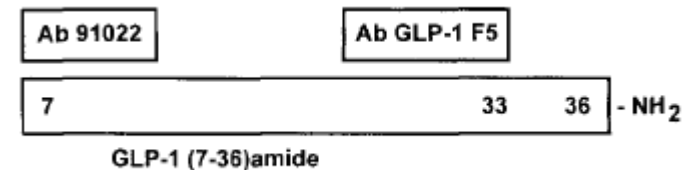
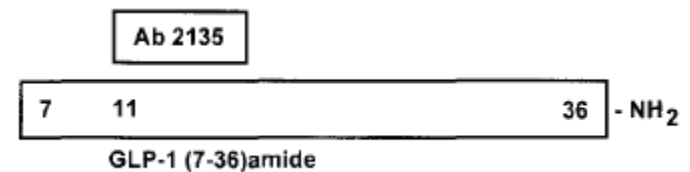
- GLP-1: endogenous 5-40 pM
- Human insulin: endogenous 50-500 pM
- The exogenous concentrations are often higher
- Immuno assays are traditionally used
- Metabolites can be agonists, antagonists and immuno reactive

# Immuno assays

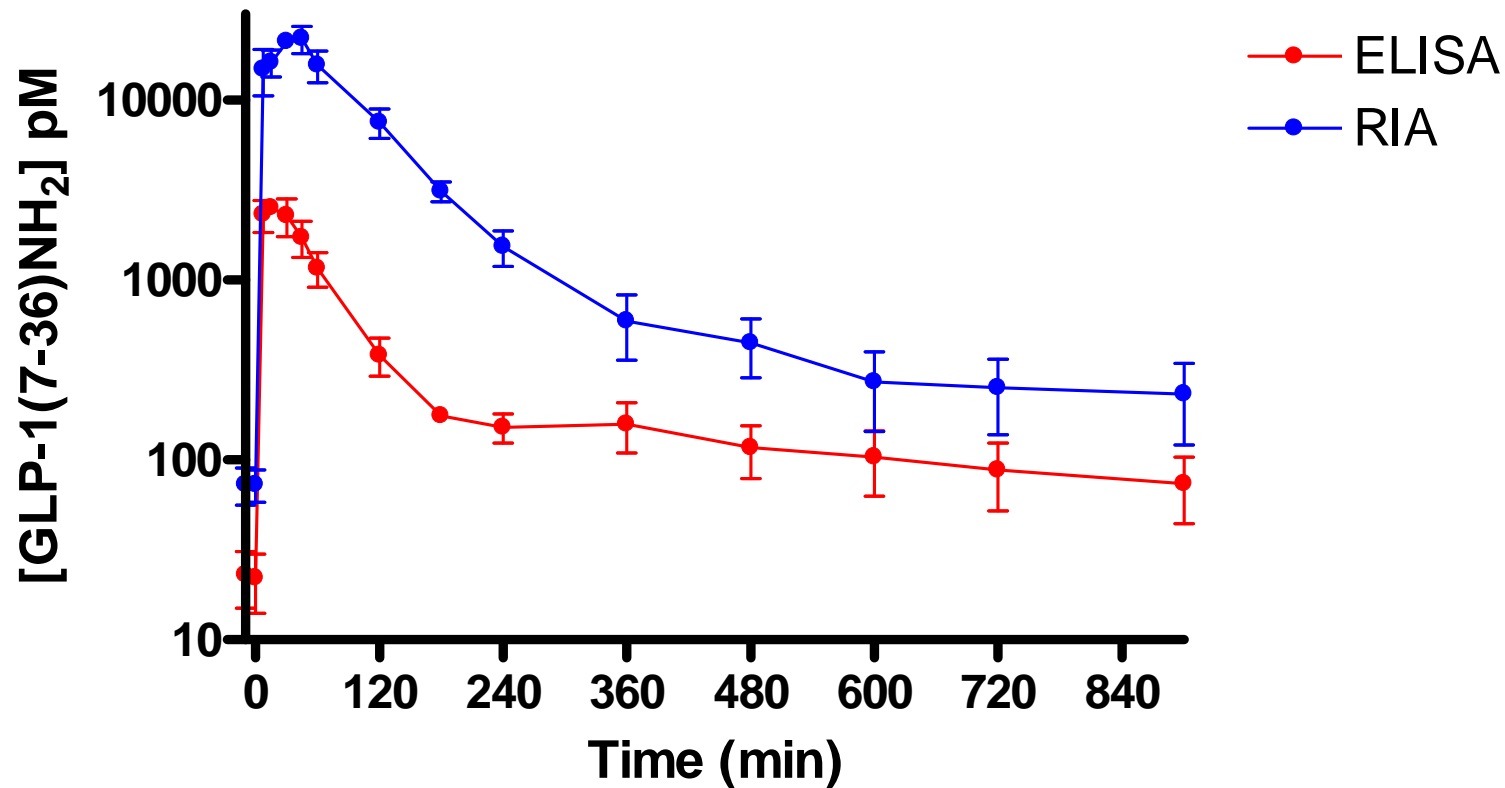
1. RIA/EIA where only one antibody is used
2. "Sandwich" assays where two antibodies bind to the peptide or protein at the same time
  - The antibody recognizes an "epitope" which consists of 10-15 amino acids
  - Immuno reactive metabolites are quantified
  - Immuno reactivity and not concentration is measured
  - It takes > 6 months to make a new antibody

# Example: GLP-1 assays

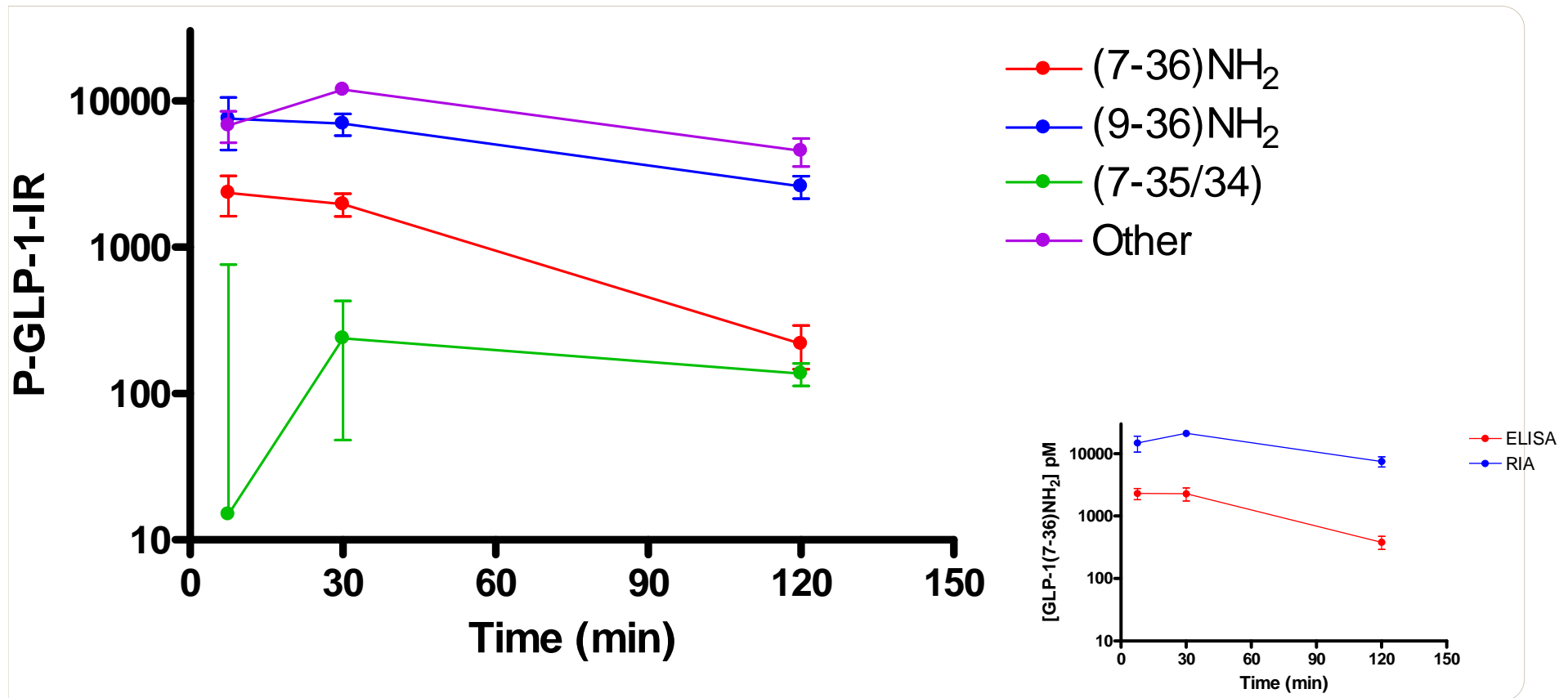
- RIA (antibody with epitope from aa 11 to aa xx)
- Sandwich ELISA (N-terminal specific antibody + antibody with epitope from aa xx to aa 33)



# 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<sup>9</sup> in the N-terminus and/or beyond Lys<sup>34</sup> in the C-terminus**

# New assay types

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

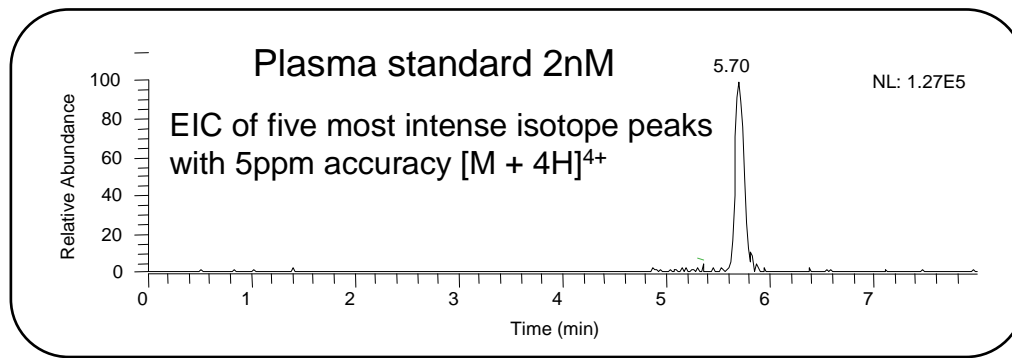
1. LCMS

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)



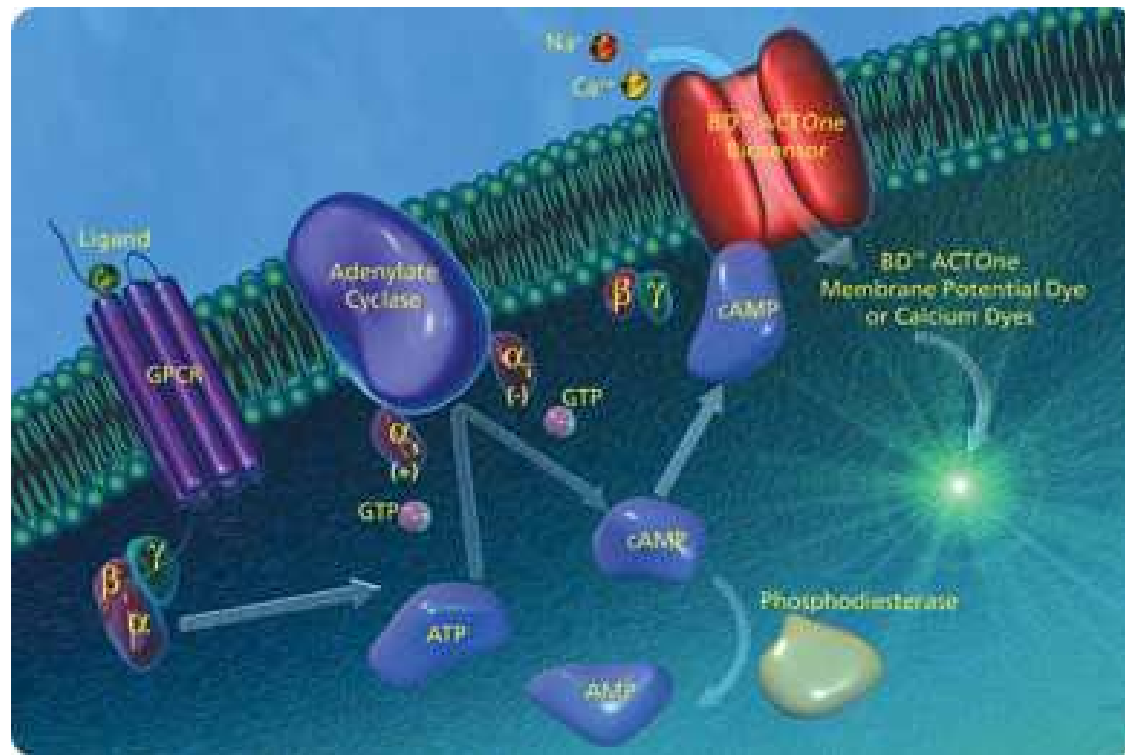
- Plasma samples are typically precipitated with organic solvent (EtOH/MeOH, Acetonitrile (1% HCOOH)). The recovery is usually >70-80%.
- In other projects, turboflow chromatography or solid phase extractions are used for sample preparation.

# 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 - **ACTOne™**



## **BD ACTOne™ cAMP Biosensor**

BD ACTOne™ is an easily scalable cAMP biosensor HTS platform for G<sub>s</sub> and G<sub>i</sub> GPCR drug discovery and deorphanization.

# Sample preparation

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

# Cell preparation

- Format: 384
- Cells: 14.000 cells/well in 25  $\mu$ l
- Over night at 37°C and 5 % CO<sub>2</sub>
- Dye: 25  $\mu$ l FLIPR® Calcium 4 Assay Kit
- 1 h at 37°C followed by 1 h at RT

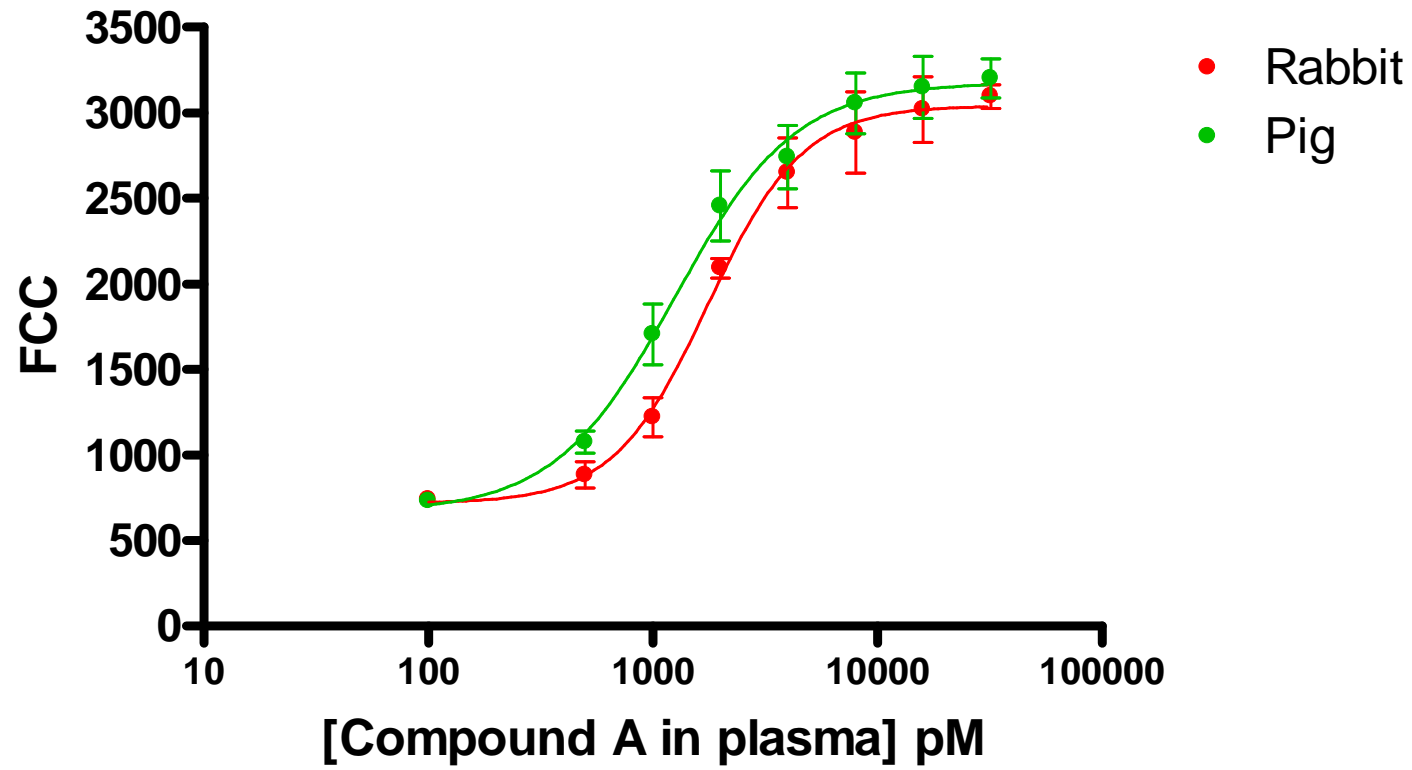
# FLIPR protocol

- $\lambda$ :
  - excitation 470-495 nm
  - emission 515-575 nm
- Timing:
  - inject  $t = 30$  sec
  - measure  $t = 360$  sec
- Volume
  - 5  $\mu\text{l}$  ( $\approx 0.8$   $\mu\text{l}$  plasma or 63 fold dilution of plasma)

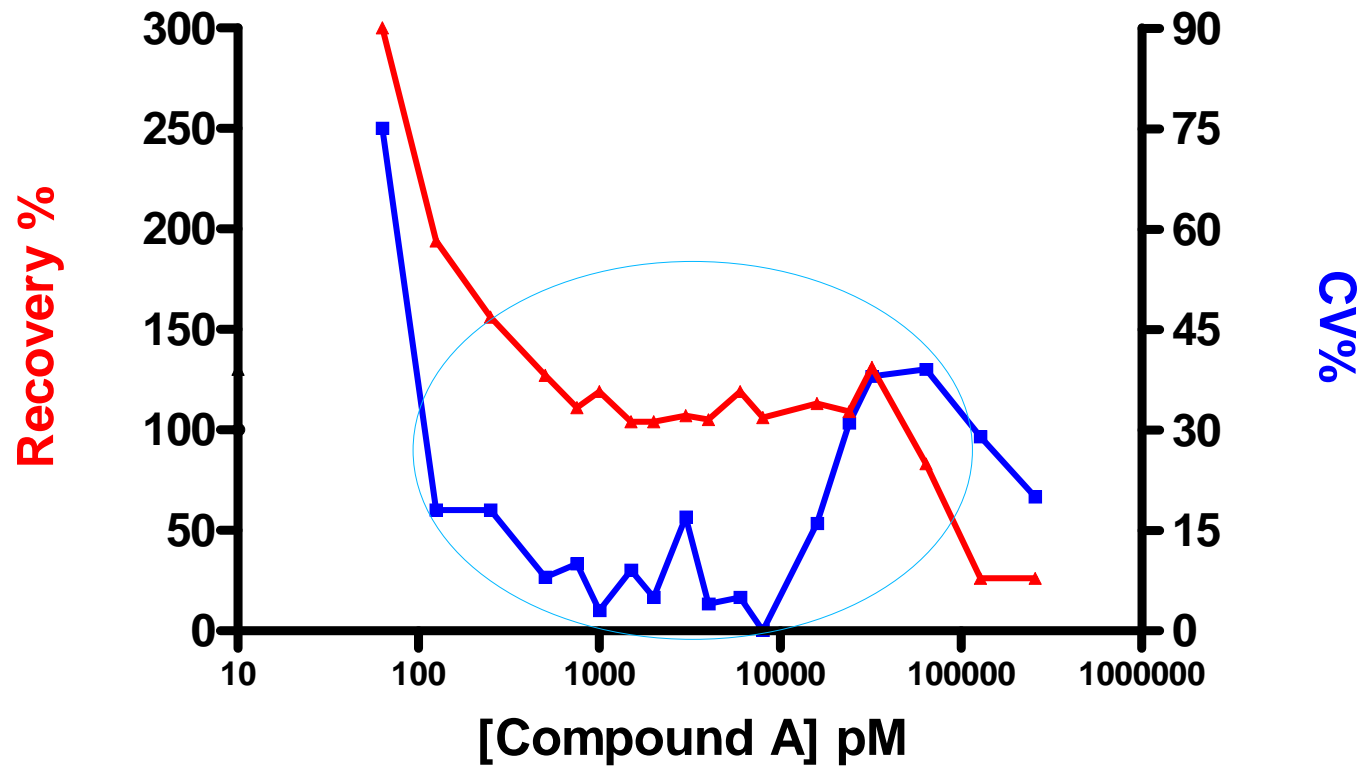
# Compound A

- 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

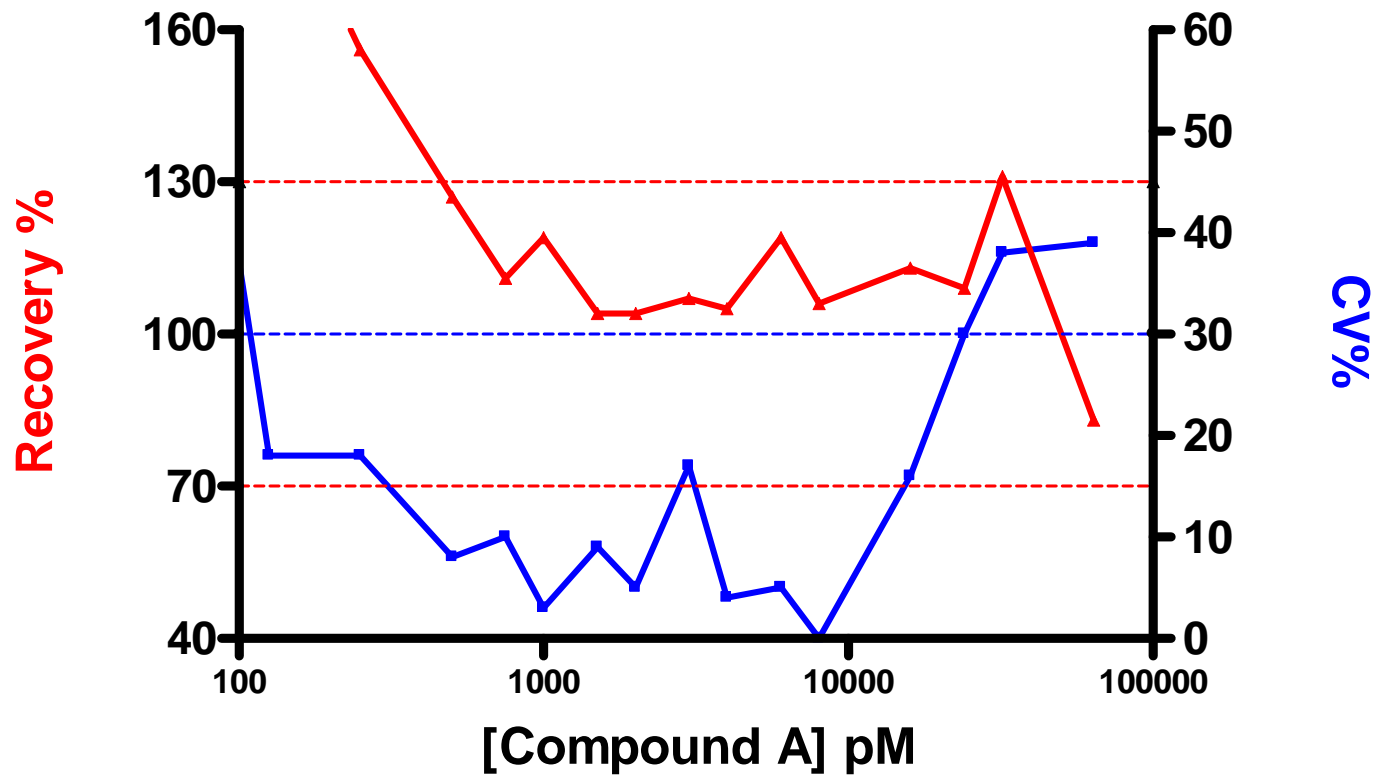
# Dependence on species



# Determination of analytical range



# Determination of analytical range



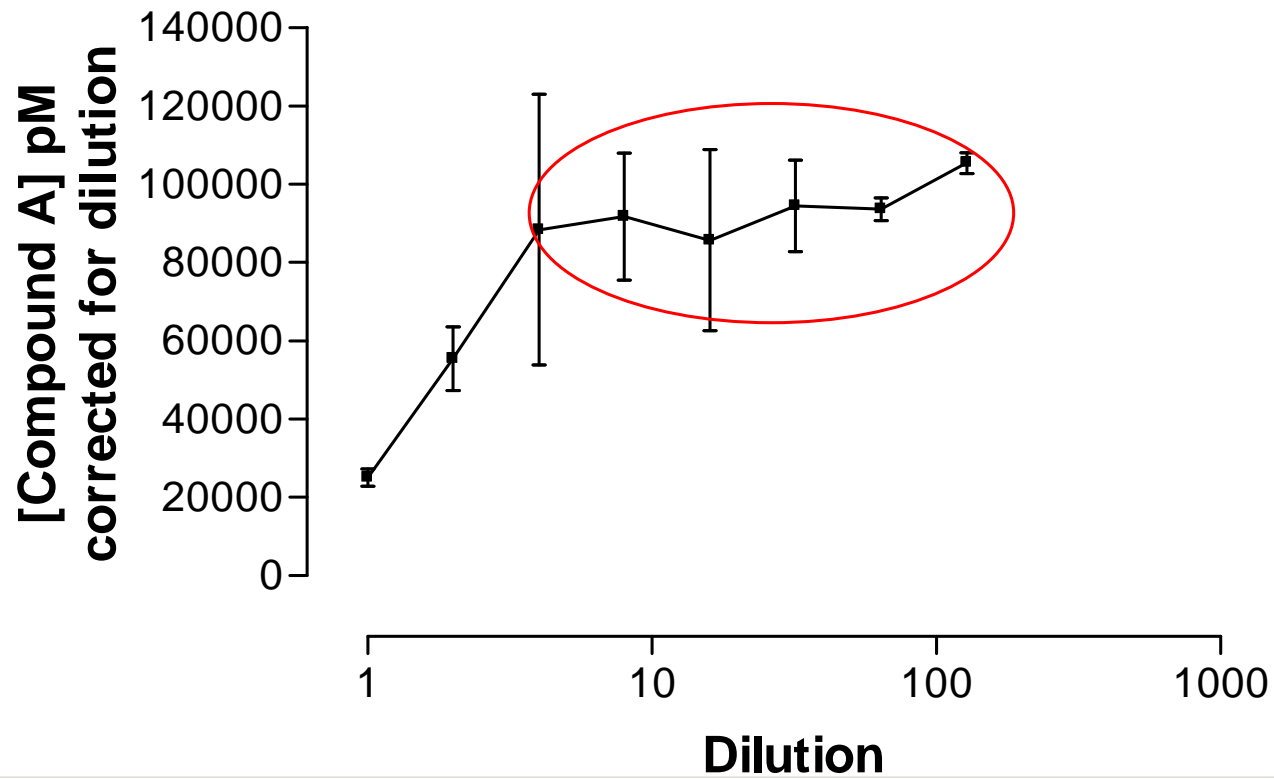
# Determination of analytical range

	LOD	LLOQ	HLOQ
1 <sup>st</sup> test	250	500	24000
2 <sup>nd</sup> 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

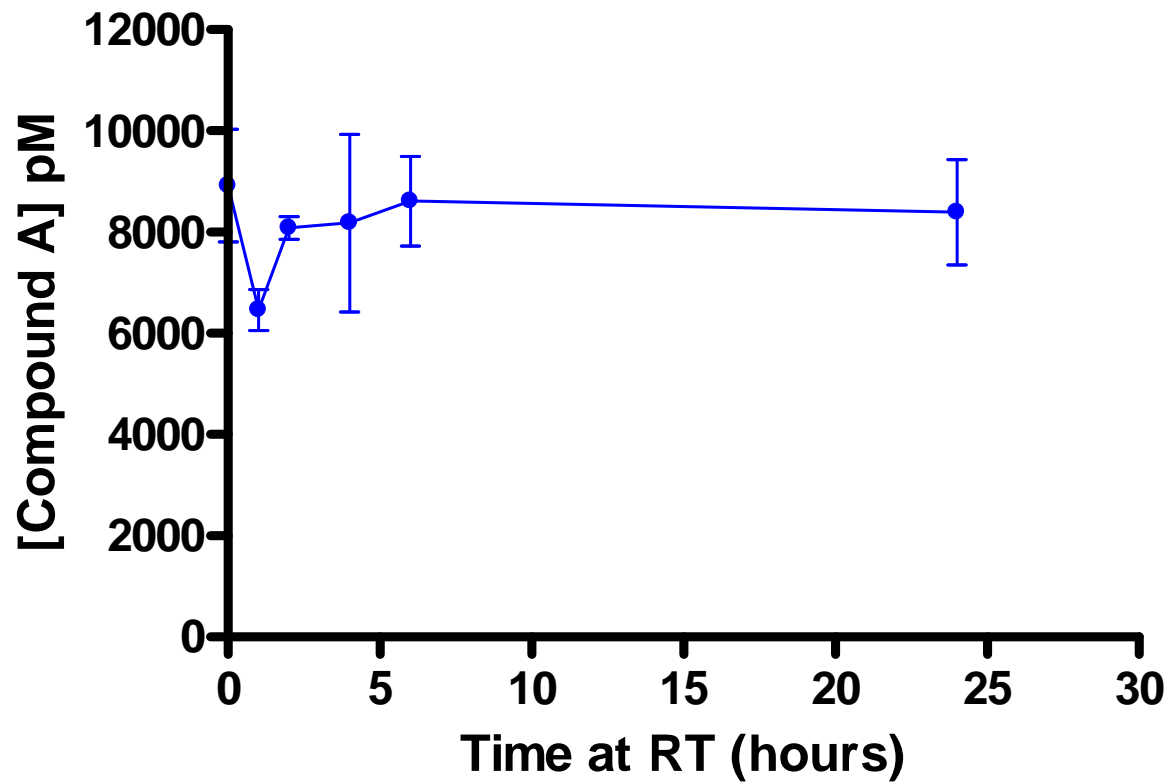
# Dilution Recovery



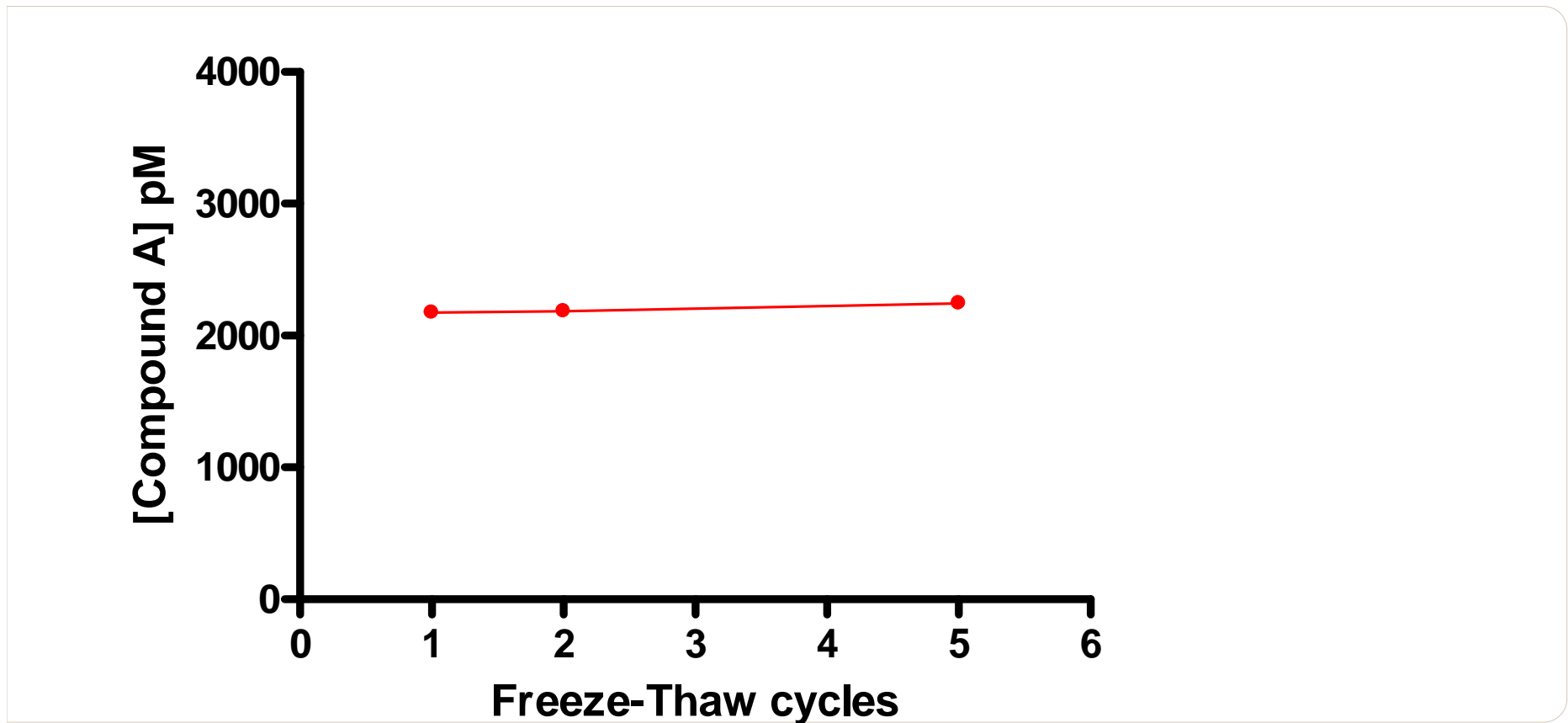
# Spiking recovery

	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

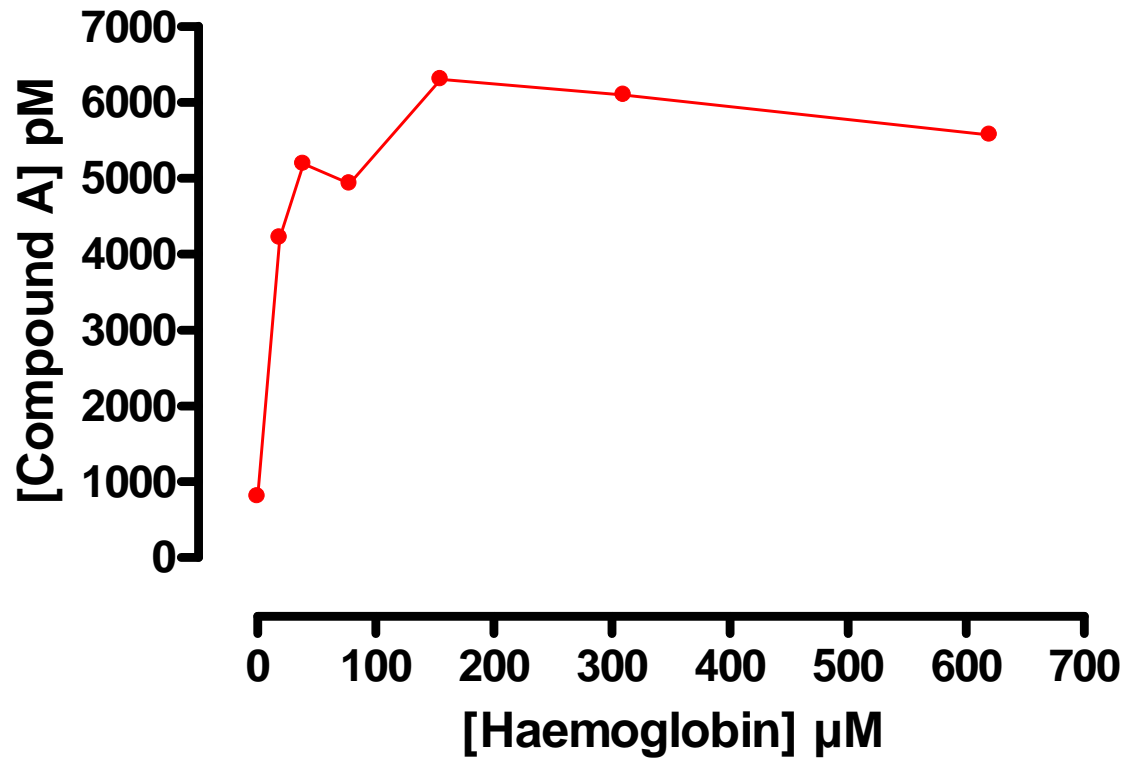
# Stability of the plasma samples



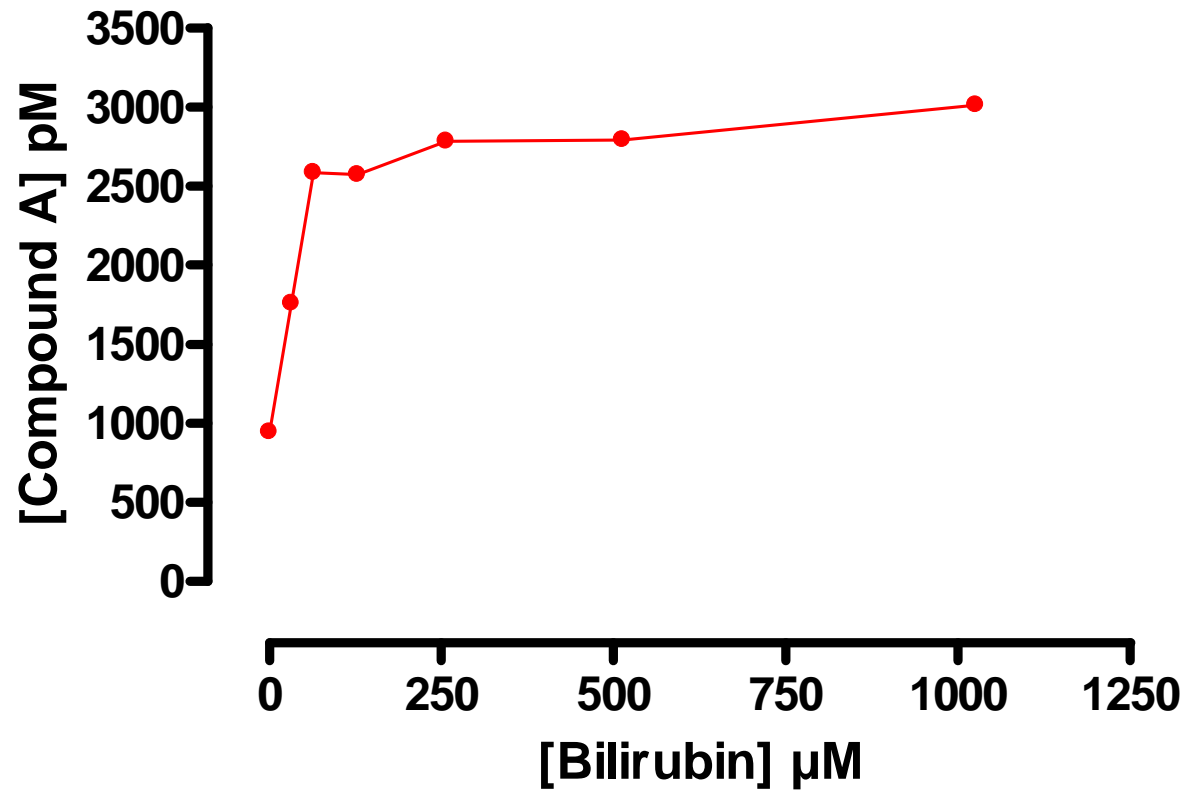
# Freeze-Thaw stability



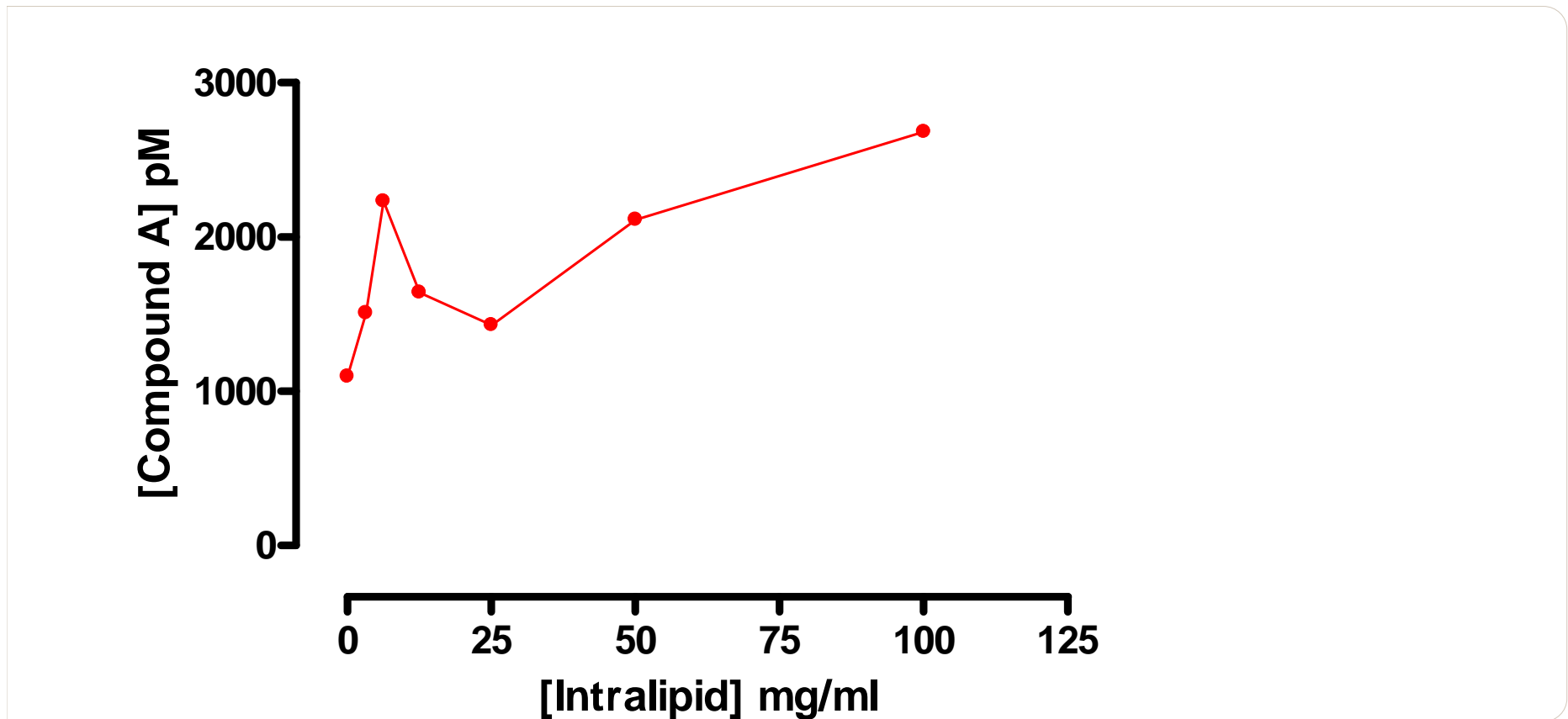
# Interference by Haemoglobin



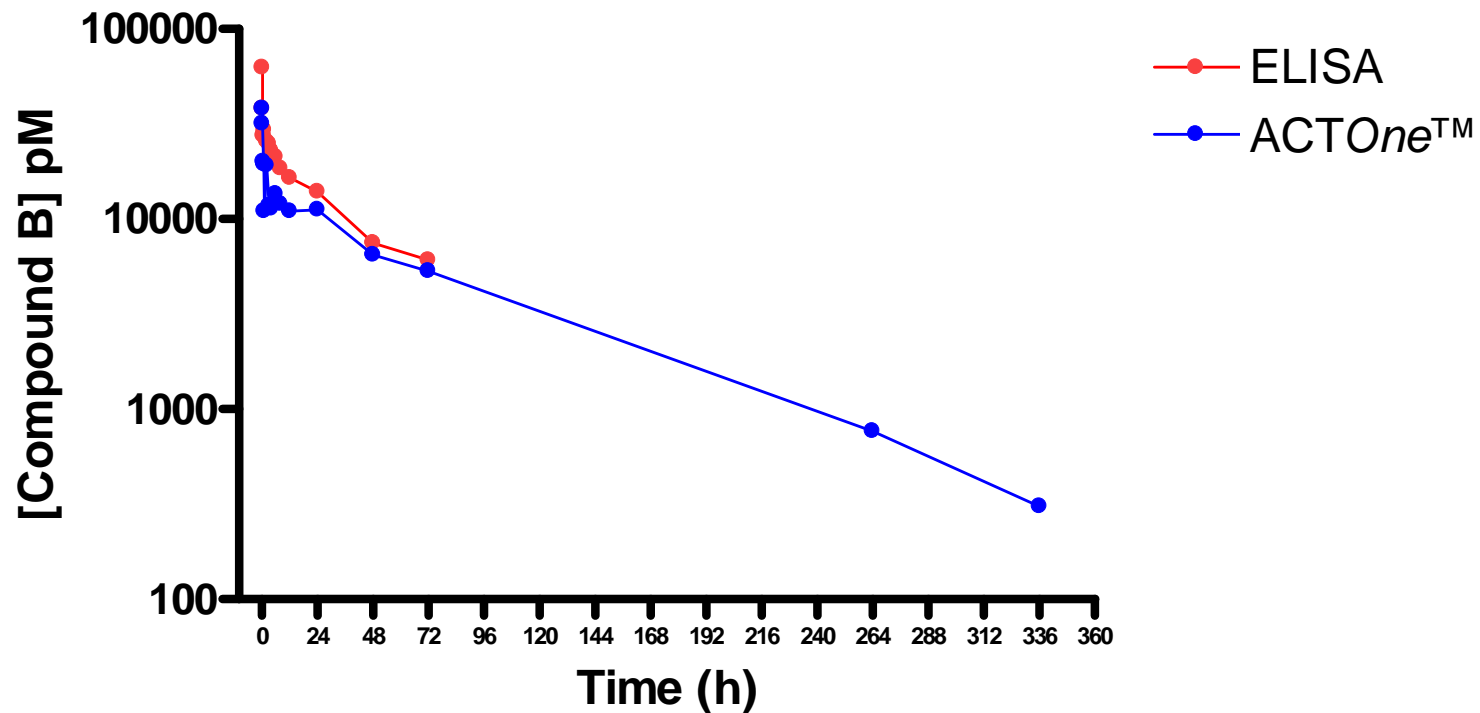
# Interference by Bilirubin



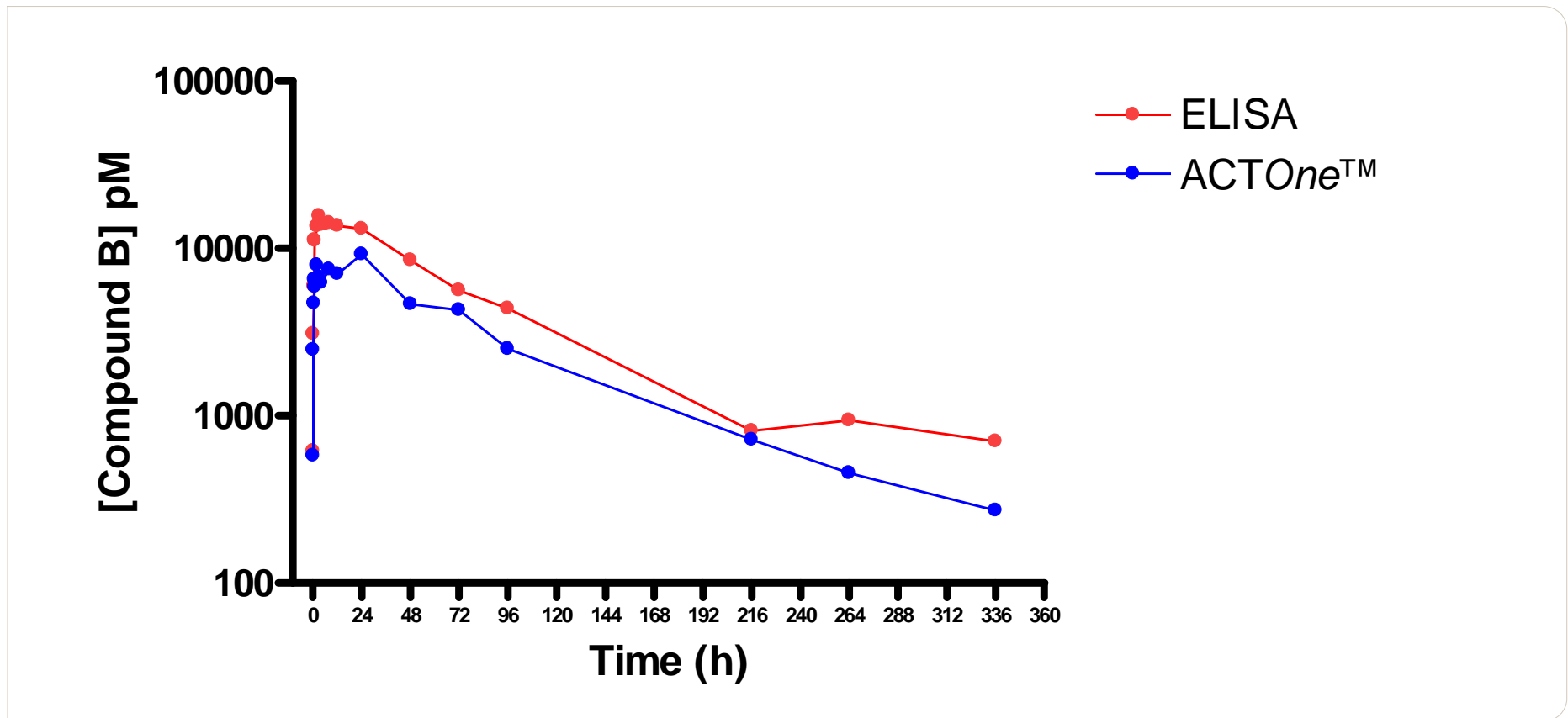
# Interference by Intralipid



# 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

<b>ASSAY</b>	<b>RESULT</b>	<b>LLOQ (pM)</b>	<b>Throughput samples/day</b>	<b>Sample Preparation</b>
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	$219 + 1.13 * 2595 + 1.48 * 137 + 4548 = 7902 \text{ pM}$
ELISA	$219 + 0.01 * 2595 + 0.90 * 137 = 368 \text{ pM}$
ACTOne	$219 - 0.01 * 2595 + 0.08 * 137 = 204 \text{ pM}$
LCMS	$219 = 219 \text{ pM}$

## Conclusion – ACTOne assay

- Sensitive assay (dependent upon potency of parent compound)
- Medium throughput
  - >3.000 samples per day ( $\approx 42 \times 384$  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
- Dr. Søren Tullin