Pictured above: HIV absorption

Tinne Huybrechts | YSS Barcelona | 17 November 2015

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Outline

- What is micro dosing and what are the benefits?
- Study design for F_{abs} studies
- Isotopic interference calculations
- Sensitive LC-MS/MS assay
- Isotopic interference in practice
- Conclusion

What is microdosing and what are the benefits?

- Micro-dose = at least 100 times lower than the proposed therapeutic dosage and a maximum dose of 100 µg
- Benefits of applying a human microdose:
  - Lower toxicological risk to subjects
  - Limited Tox package to go into man
  - Requires less drug substance
- High sensitivity required of bioanalytical assay (~ pg/ml level) to allow generating full profile
- In the past these levels were only achievable by Accelerator Mass Spectrometry (AMS) after dosing ^{14}C-labeled compound
- AMS remains unbeatable in sensitivity but also expensive and complex
- With the sensitivity improvements of newer mass specs LC-MS/MS is becoming a viable alternative

The study design for F_{abs} study

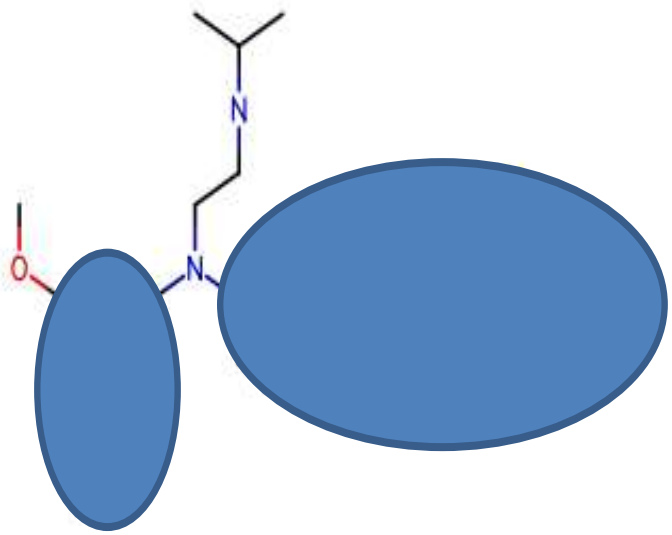
PO dose = 10 mg

IV dose = 100 µg

- Analysis of the therapeutic (PO) dosed NL-compound
  - Regulated validated assay available
  - 0.5 ng/ml – 500 ng/ml
  - IS concentration: 10 ng/ml
- Analysis of the µD-compound (IV)
  - Scientific validated assay
  - Range: 1 pg/ml – 1 ng/ml
  - IS concentration: 1 ng/ml
- **Selection of a suitable stable isotope labeled µD-compound!!**

Isotopic calculations – The structures

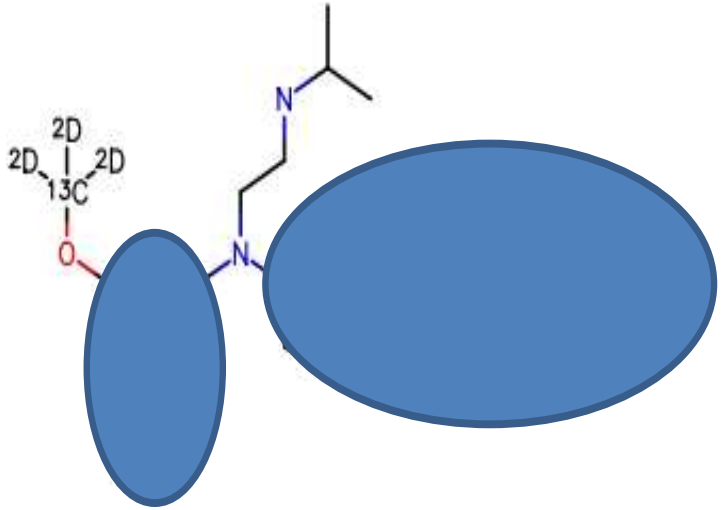
NL-compound



Exact Mass: 446.243

M

STIL-IS



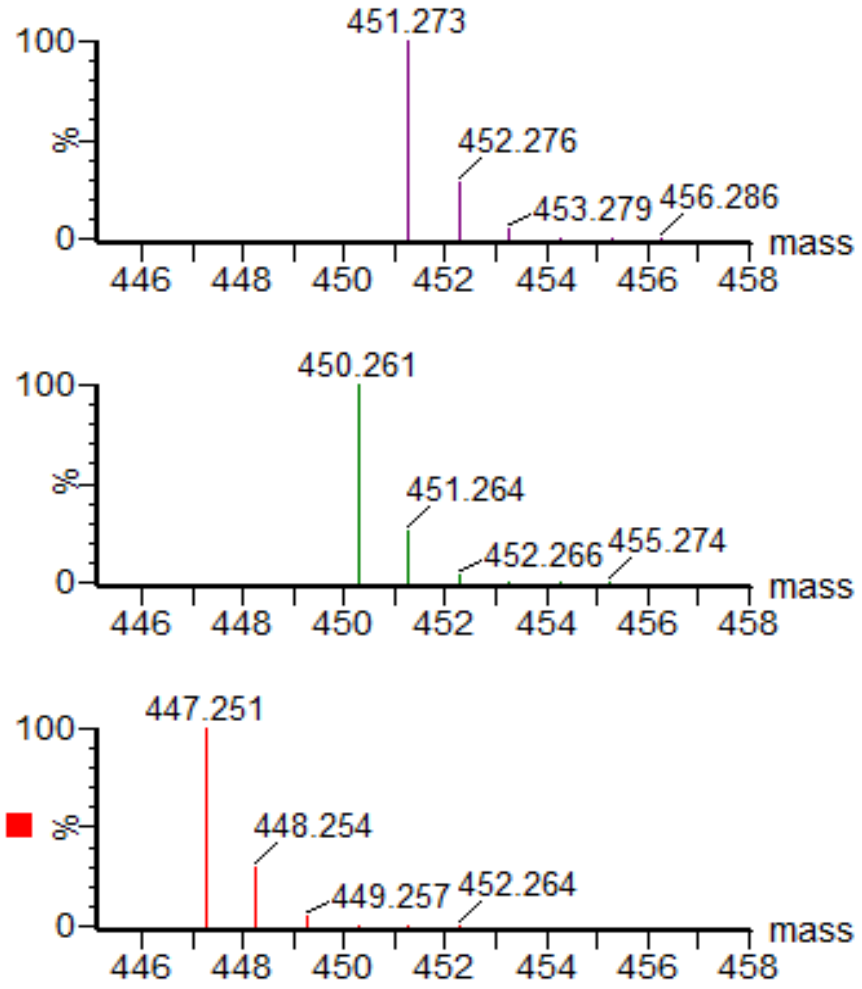
Exact Mass: 450.265

M+4

Isotopic calculations – Isotopic distribution of NL-compound

$(M+H)^+$ of NL-compound

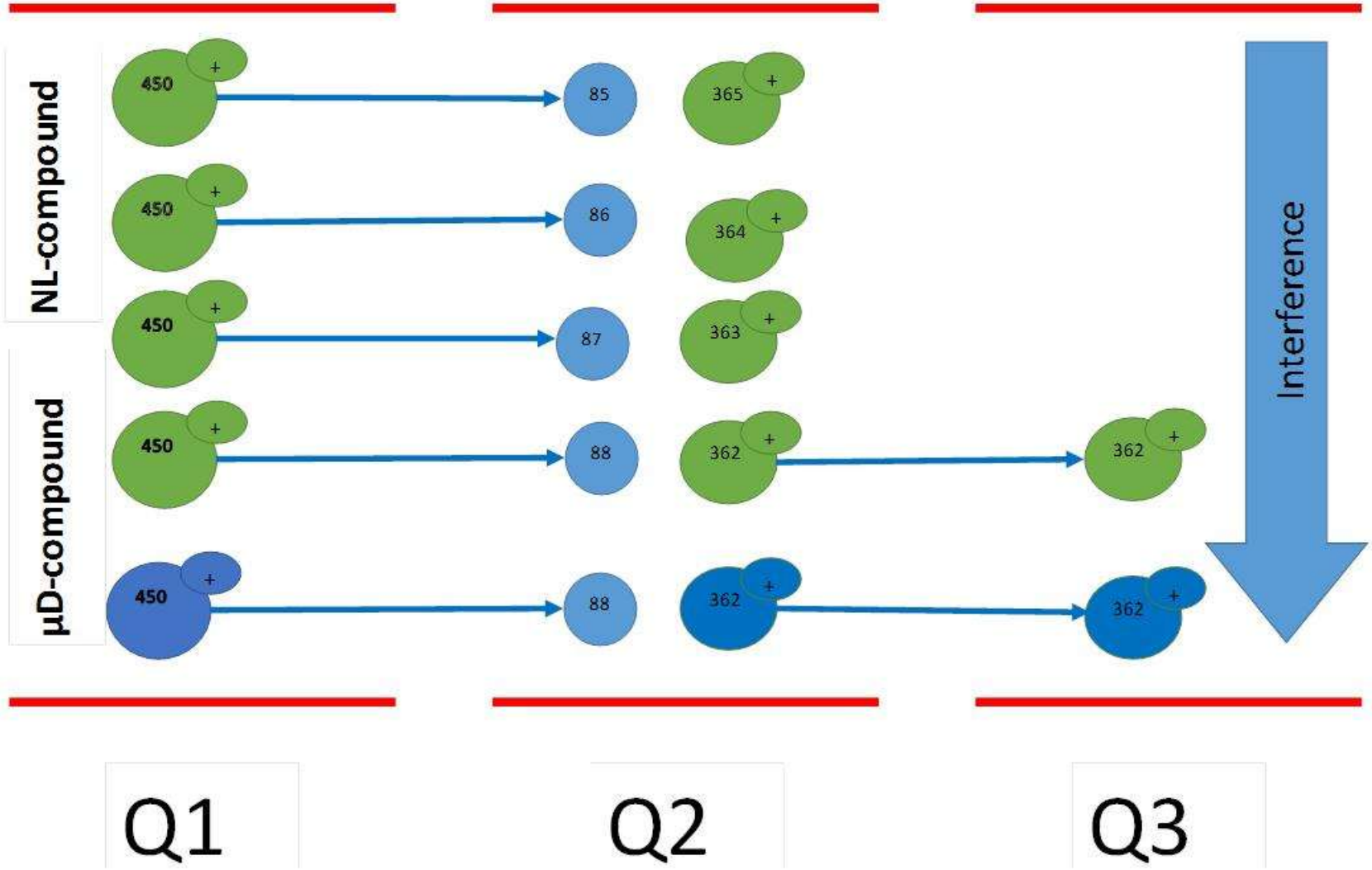
	Parent mass	Abundance %	% IF (at 100 fold higher conc of NL-cpd)
0	447	100	
1	448	29.7834	2978
2	449	4.686	469
3	450	0.5159	51.6
4	451	0.0439	4.39
5	452	0.003	0.3
6	453	0.0001	0.01



Isotopic calculations – Isotopic ditribution of NL-compound

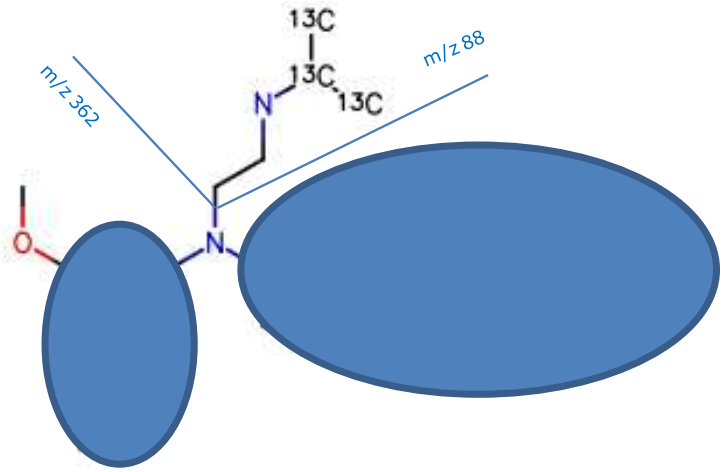
- Interference calculations are overestimated since fragmentation is not taken into account
- **Fragmentation impact can further reduce isotopic interference**
- Calculation and mitigation of isotopic interferences in liquid chromatography – Mass spectrometry/mass spectrometry assays and its application in supporting microdose absolute bioavalibility studies (Gu et al. Analytical chemistry 84,4844-4850, 2012)

Isotopic calculations
MRM transitions



Isotopic calculations MRM transitions

µD-compound
450.2/362.0



# labels on PI fragment	Parent	Neutral loss fragment		Product ion (PI) fragment			
	mass	mass	abundance (%)	mass	abundance (%)	Combined abundance (%)	% IF (at 100 fold higher conc of NL-cpd)
3	450	85	100	365	0.293	0.29	29
2	450	86	5.95	364	3.12	0.19	19
1	450	87	0.147	363	23.83	0.035	3.5
0	450	88	0.0019	362	100	0.0019	0.19

Sensitive LC-MS/MS assay – Method development

- Range: 1pg/ml – 1ng/ml
- Sample preparation
  - Supported liquid extraction (SLE+)
  - 200µl plasma + 20µl internal standard + 100µl NaOH
  - Extract with 2 x 0.5 mL TBME
  - Evaporate
  - Reconstitution: 100µl MeOH + 100µl $(NH_4)_2CO_3$
- LC-conditions
  - Flow: 600µl/min
  - Column: 50mm x 2.1 mm I.D., packed with 1.7µm Acquity UPLC BEH C18 @ 50° C
  - QTRAP 6500 + Nexera
  - Injection volume: 10µl

Time (min)	10 mM $(NH_4)_2CO_3$	CH_3OH
0	40	60
2.0	10	90
2.01	2	98
3.0	2	98
3.01	40	60

Isotopic interference – in practice

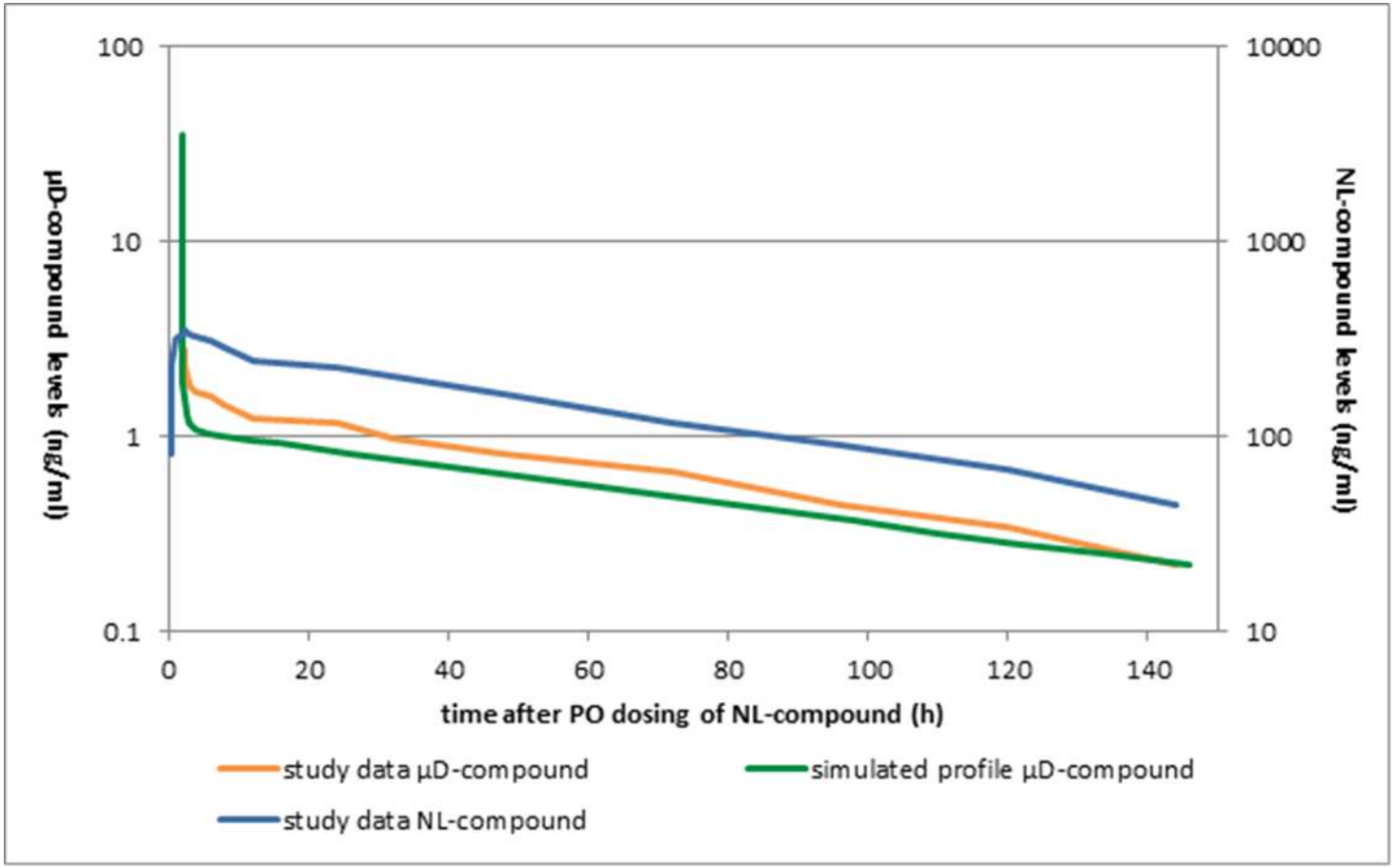
- Evaluation contribution NL-compound to µD-compound
  - Quantification of the interference from human plasma samples containing excess NL-compound

Plasma sample NL-cpd		Plasma sample µD-cpd				
Conc NL-cpd (ng/ml)	PA µD-cpd	Conc µD-cpd (pg/ml)	PA µD-cpd	% contribution	factor	% theoretical contribution
20	1587	200	1121953	0.14	100	0.19
400	20943*	1000	5579543	0.38	400	0.76

* Ion suppression on the signal of µD-compound in samples containing excess NL-compound

➢ samples with high NL-cpd concentrations, exceeding the ULOQ conc of the µD-cpd assay by more than 100-fold, should be analyzed after dilution

Predicted IV profile vs. Mean PO and IV study data



Isotopic interference in microdose studies - conclusions

- Theoretical evaluation of the isotopic interference drives the selection of labels in µD-compound
- Observed and theoretical isotopic contributions match
  - Samples with high NL-compound concentrations should be analyzed after dilution to avoid ion suppression effects
- Ratio NL-compound/µD-compound needs to be evaluated to make sure that isotopic interference remains insignificant – prediction tools are useful
- Isotopic interference on STIL-IS needs to be considered as well
- Method scientifically validated[1] (poster presentation)
- Study design included QC samples in presence of NL-compound

[1]: P. Timmerman, S. White, S. McDougall, M. A. Kall, J. Smeraglia, M. Scheel, M. Knutsson. "Tiered approach into practice: scientific validation for chromatography-based assays in early development - a recommendation from the European Bioanalysis Forum. Bioanalysis 2015, 7(18), 2387-2398

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