NUVISAN

Combined LC-MS/MS method for a highly instable small molecule and its two metabolites – complication and troubleshooting

9th YSS (11/12-May-2023)

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Overview

- Project scope
- Chromatographic challenges
- Stability issues of the parent molecule
- Approaches to improve stability
- Validation overview and results
- Challenges during animal study
- Conclusion

Project scope

Analytical method: Liquid chromatography with tandem mass spectrometry

Matrix: rat whole blood

Ranges:

0.100 – 200 µg/L blood (parent molecule)

10.0 – 20000 µg/L blood (carboxylic metabolite)

1.00 – 2000 µg/L blood (alcohol metabolite)

Chromatographic challenges

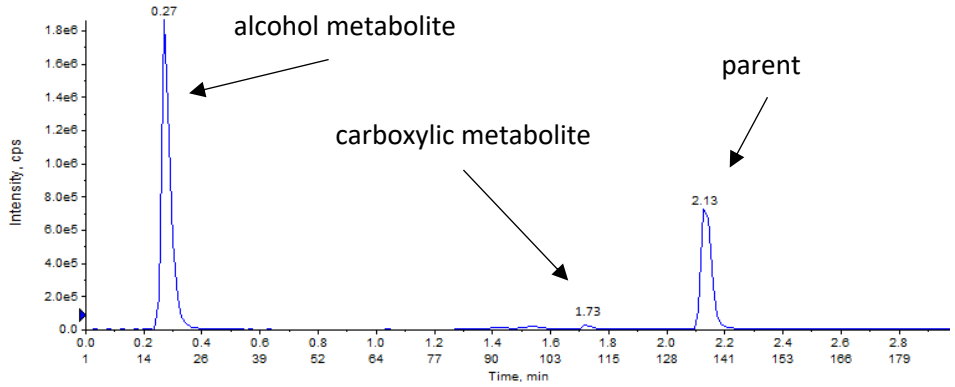
Aim: 3 molecules, one method (best case)

But: different chemical properties

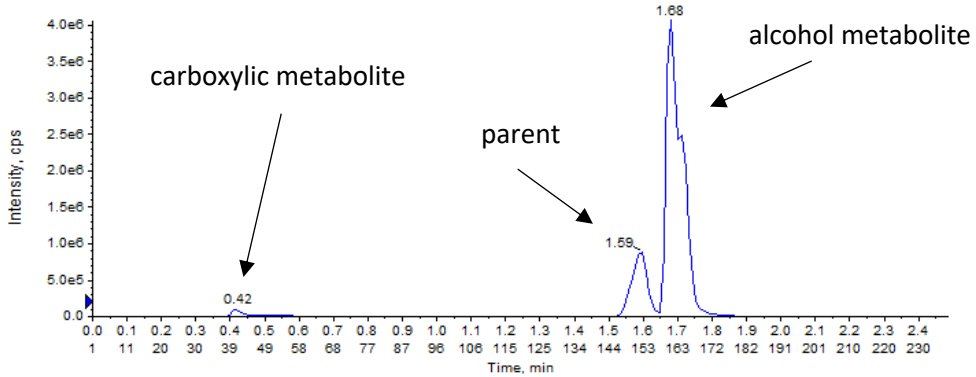
- Alcohol metabolite more hydrophobic
- Parent and carboxylic metabolite more polar

→ two separate methods

C18 column



HILIC column

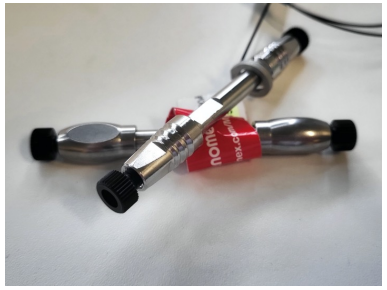


Chromatographic challenges

Method A:

Parent molecule and carboxylic metabolite

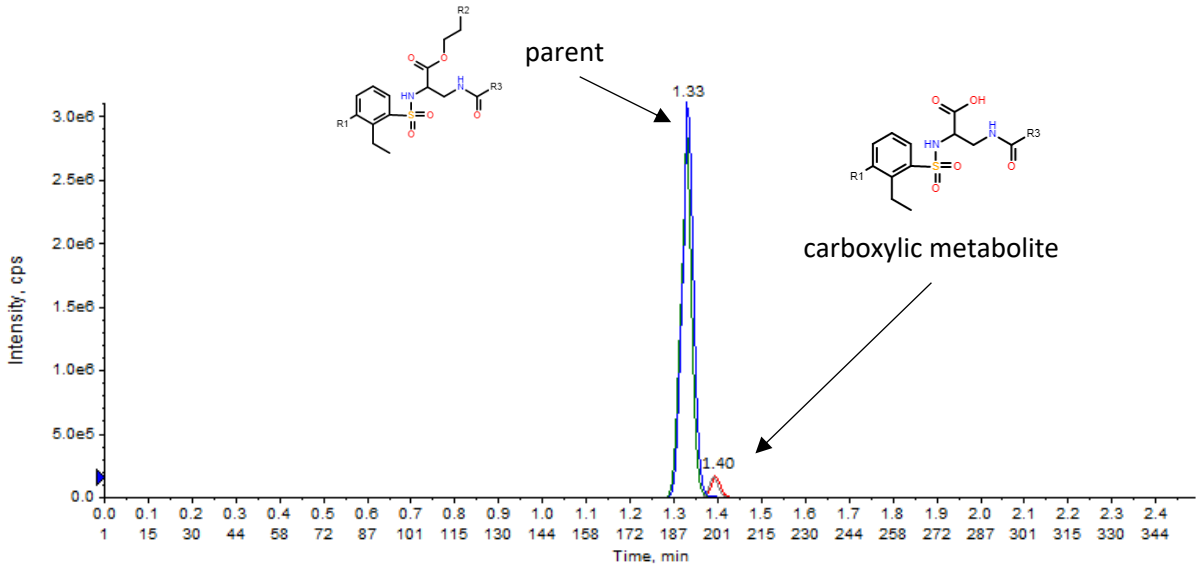
- Reverse phase C18 column
- Elution solvent composition water/acetonitrile/ammonium formate/ formic acid

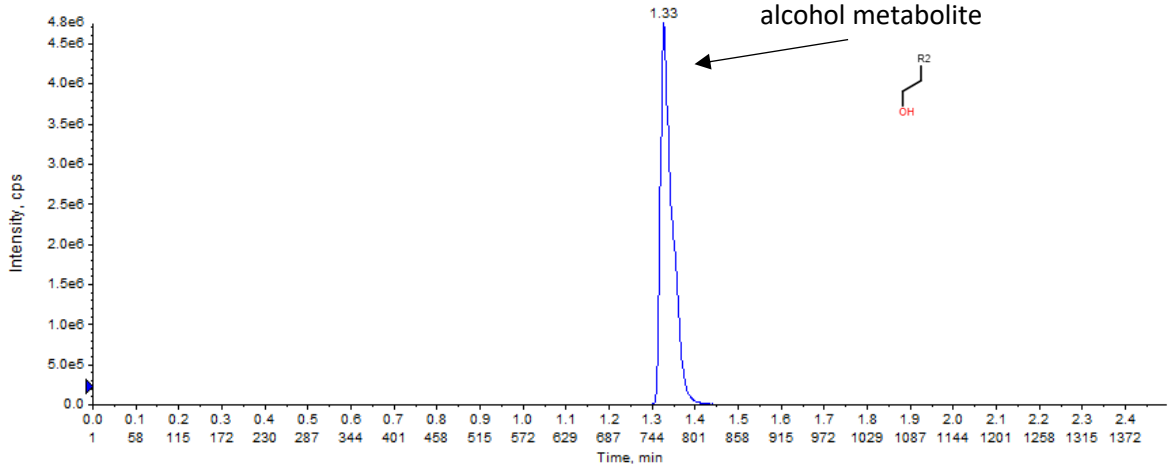


Method B:

Alcohol metabolite

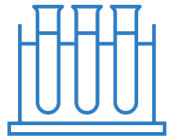
- HILIC column
- Elution solvent composition water/acetonitrile/ammonium formate/ formic acid



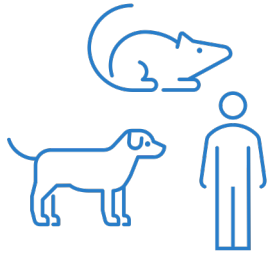


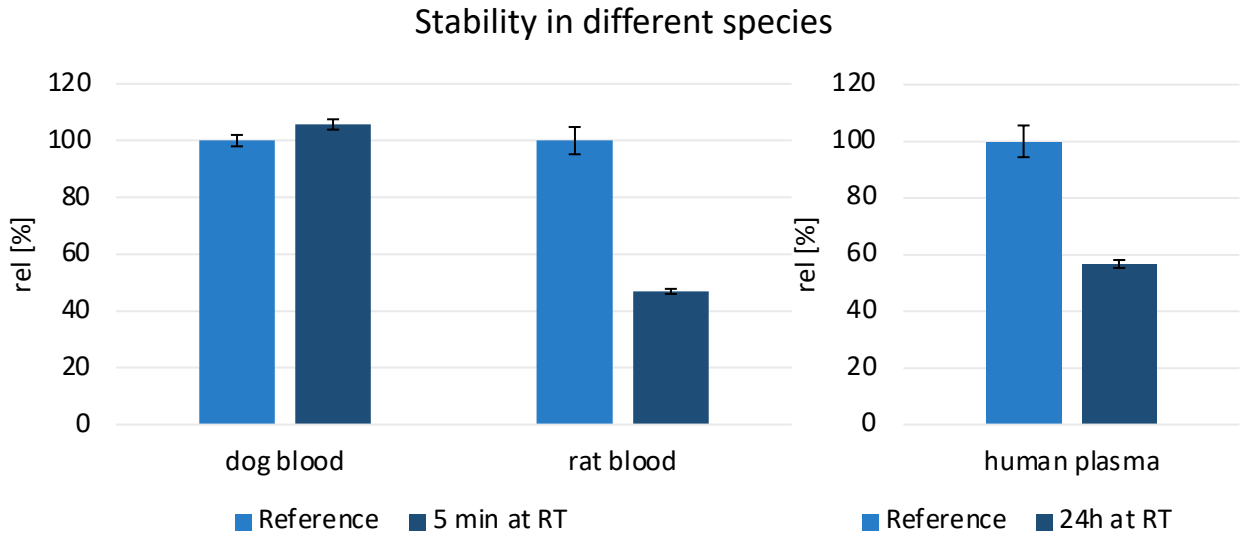
Stability issues of the parent molecule

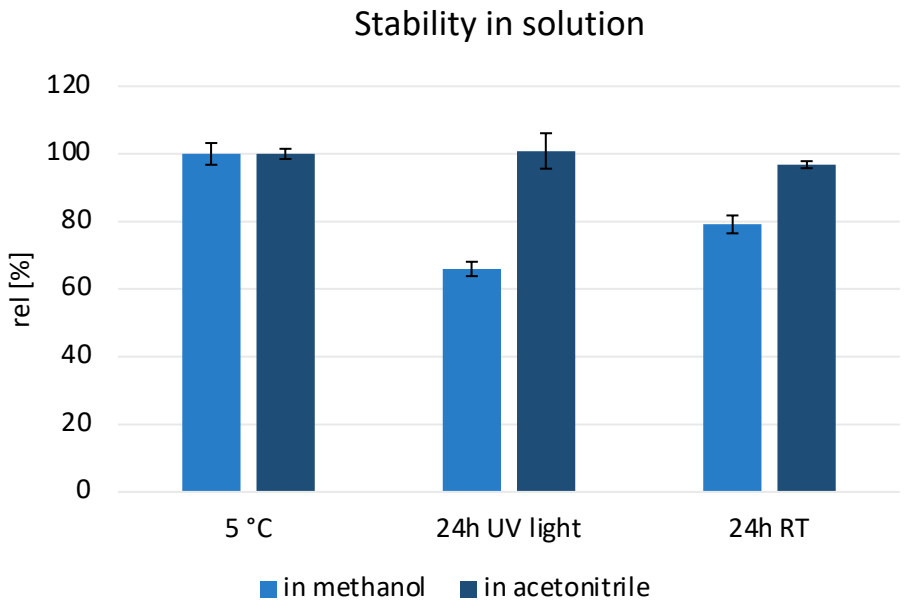
- Instability in solution



- Enzymatic instability within rat, dog and human



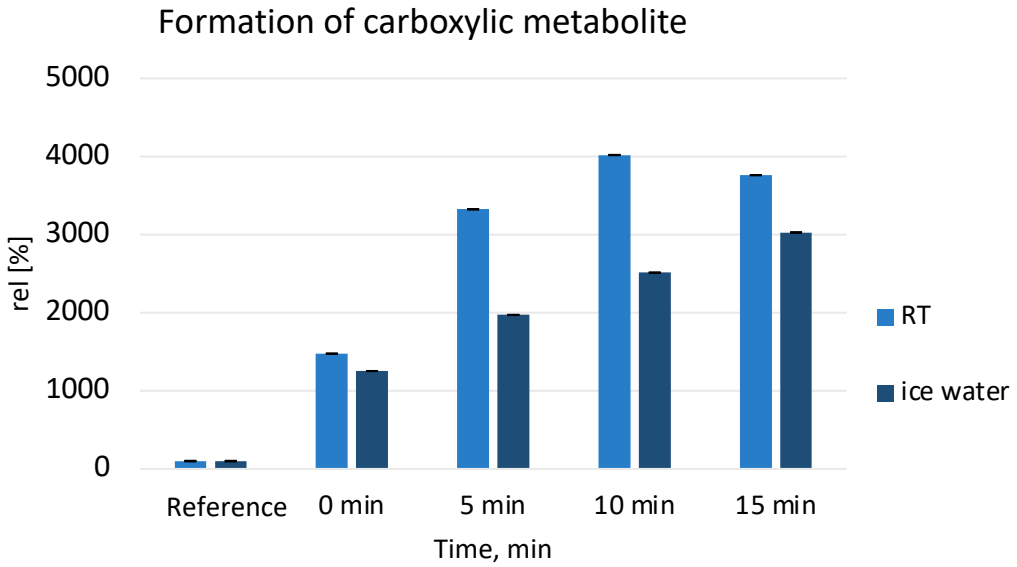


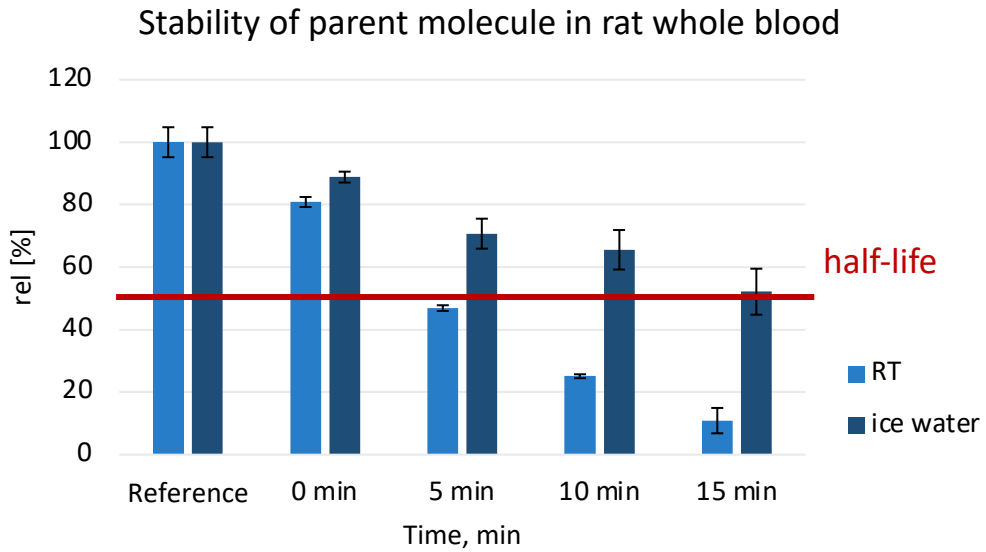


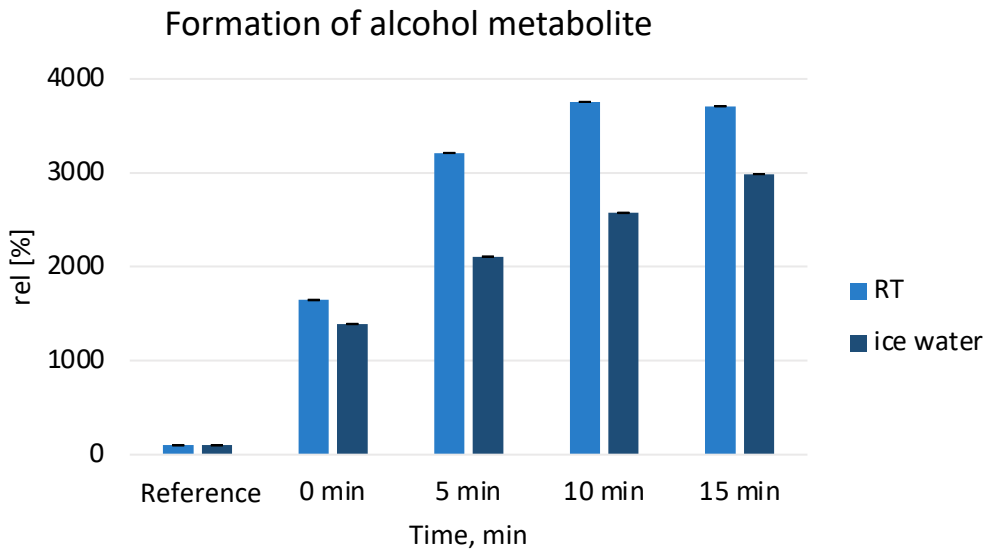
Stability in rat whole blood

Half-life of parent molecule: approx. 5 min at room temperature

- Ice water slows down enzymatic metabolism
- Half-life of approx. 15 min at ~4 °C

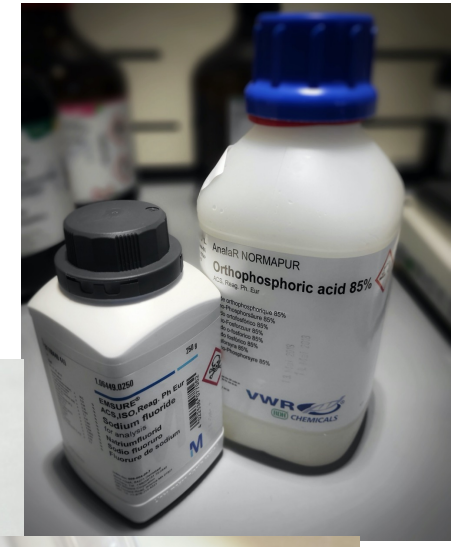


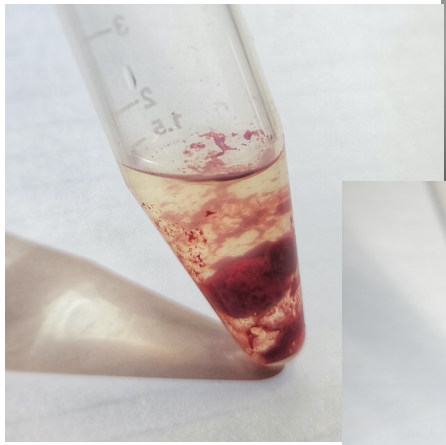


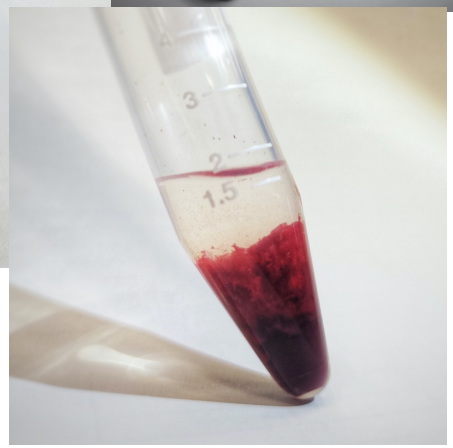


Approaches to improve stability

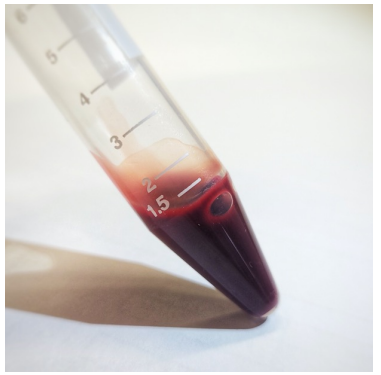
1. Addition of 1.50 mg/mL sodiumfluoride
2. Addition of phosphoric acid
3. Precipitation of blood with acetonitrile







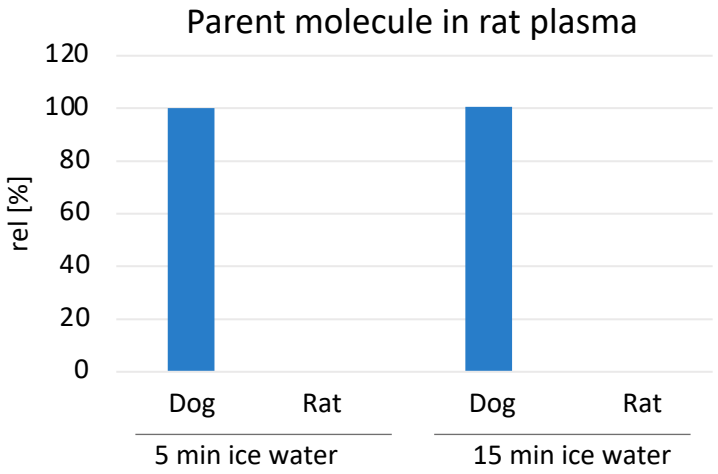
1. Addition of Sodium fluoride

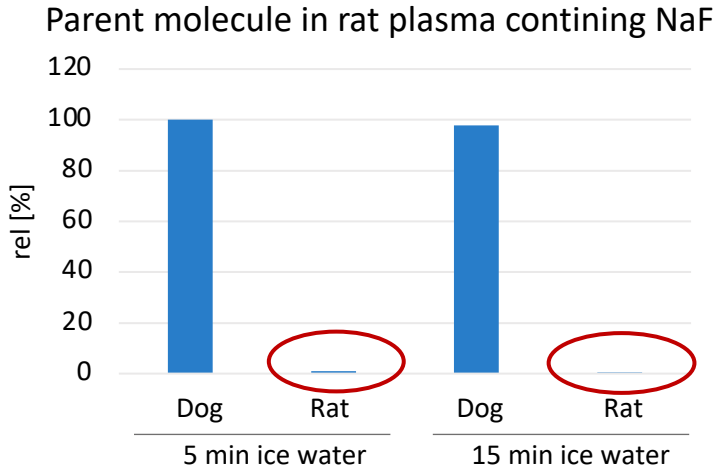


- Addition of 1.5 mg/mL sodium fluoride
- Dog plasma serves as reference
- Very low effect of NaF on stability within rat plasma

Parent molecule	without NaF			
	5 min ice water		15 min ice water	
	dog plasma	rat plasma	dog plasma	rat plasma
n	3	3	3	3
mean	6.05	0.00	6.09	0.00
cv [%]	3.1	-	6.5	173.2
rel [%]	100.0	0.0	100.6	0.0

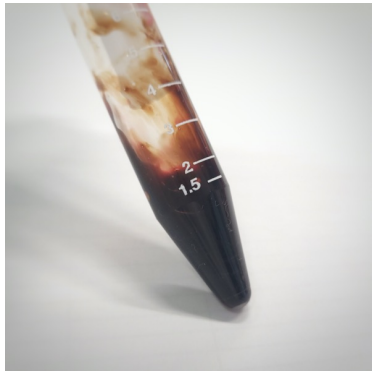
Parent molecule	with NaF			
	5 min ice-water		15 min ice-water	
	dog plasma	rat plasma	dog plasma	rat plasma
n	3	3	3	3
mean	5.77	0.05	5.64	0.02
cv [%]	1.4	87.4	1.7	11.1
rel [%]	100.0	0.9	97.7	0.4

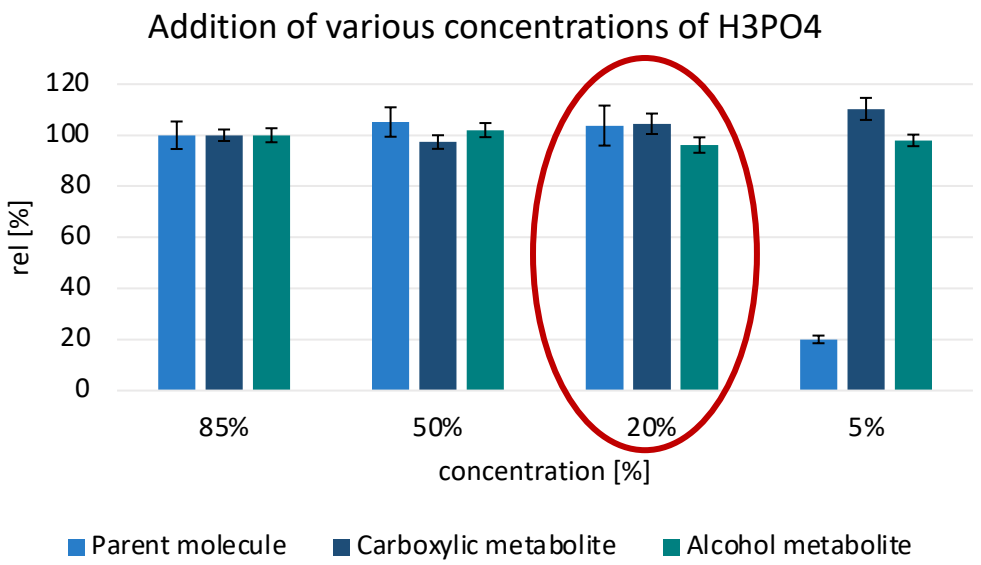


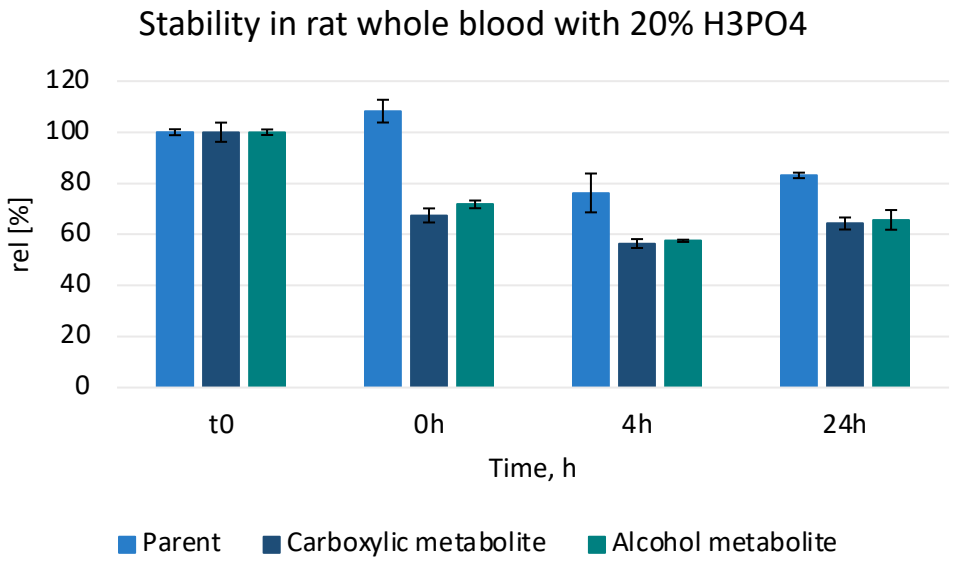


2. Addition of phosphoric acid

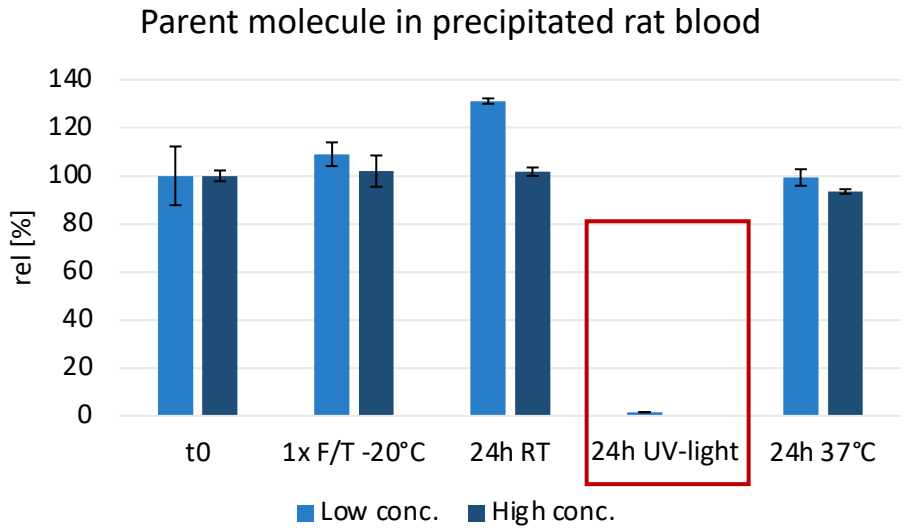
- Concentrations <20% of phosphoric acid may stabilize the parent molecule in rat blood
  - ➢ **but** coagulation and jellification of blood
- Poor stabilization at concentrations lower 20% phosphoric acid

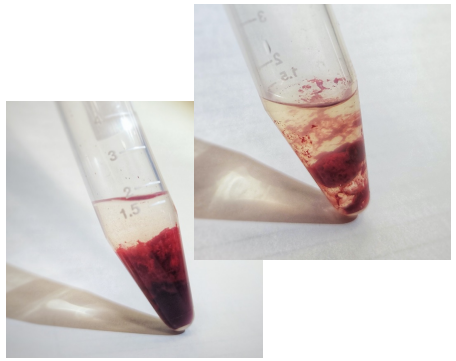


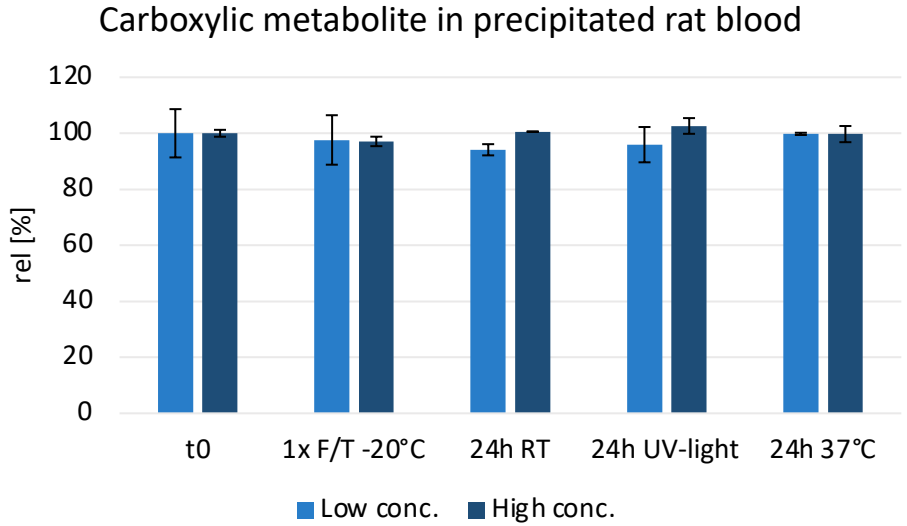


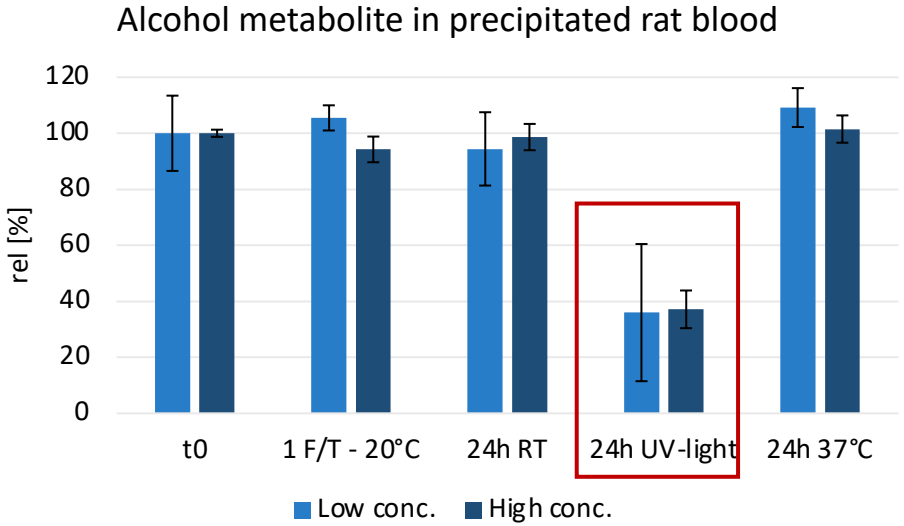


3. Precipitation of rat whole blood with acetonitrile









Final Method

Matrix: precipitated rat whole blood

- Precipitation of blood with acetonitrile
- Adaption of concentration ranges

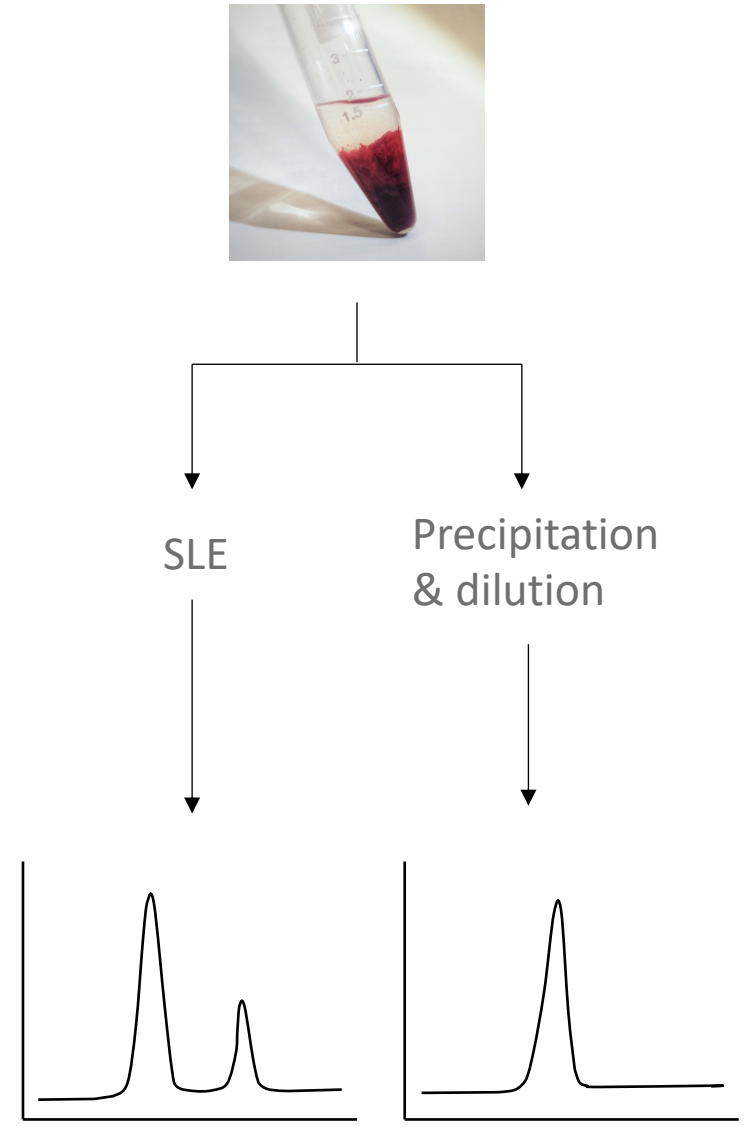
  0.0250 – 50.0 µg/L (parent molecule)

  2.50 – 5000 µg/L (carboxylic metabolite)

  0.25 – 500 µg/L (alcohol metabolite)

- Different sample preparations
  - Supported liquid phase extraction for parent and carboxylic metabolite
  - Protein precipitation and dilution for alcohol metabolite

- Different chromatographic methods

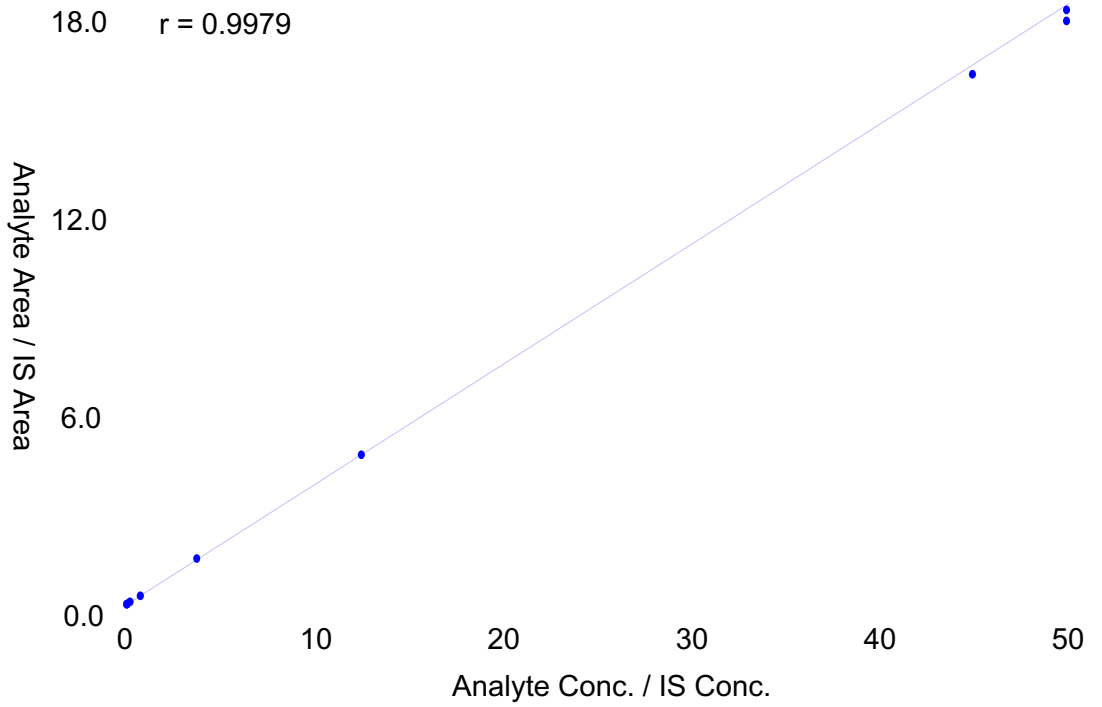


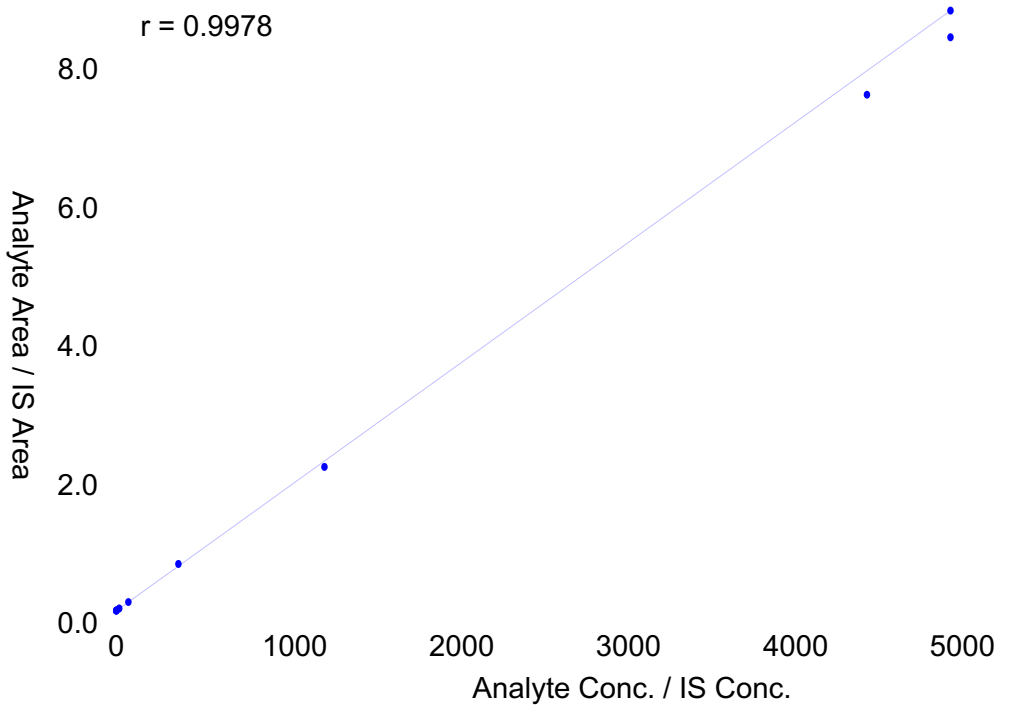
Validation overview

Validation item	Parent & carboxylic metabolite	Alcohol metabolite
Accurancy & Precision	Intra-Run Inter-Run	
Selectivity	In pooled matrix In individual matrix	
Dilution Integrity	Dilution factor 10	
Carry-over (<20% LLOQ)	#2 blank	#1 blank
Matrix Factor	1.041 (parent), 1.048 (carboxylic metabolite)	0.970
Recovery	60 % (parent), 73 % (carboxylic metabolite)	95%
Stability solutions	24 h at ambient temperature 42 days at 5 °C	24 h at ambient temperature 86 days at 5 °C
Stability in matrix (benchtop)	4 h at RT without protection from daylight 24 h at RT with protection from daylight	24 h at RT without protection from daylight
Stability in matrix (Freeze &Thaw)	6 cycles: -20°C and -75°C between cycles	
Stabiliy of processed samples (autosampler)	75 h at 10 °C	80 h at 10 °C
Stability Long-Term-Storage	Up to 6 months at -20 °C and -75°C	

Validation results

Linearity of calibration curve and accuracy and precision of QC samples



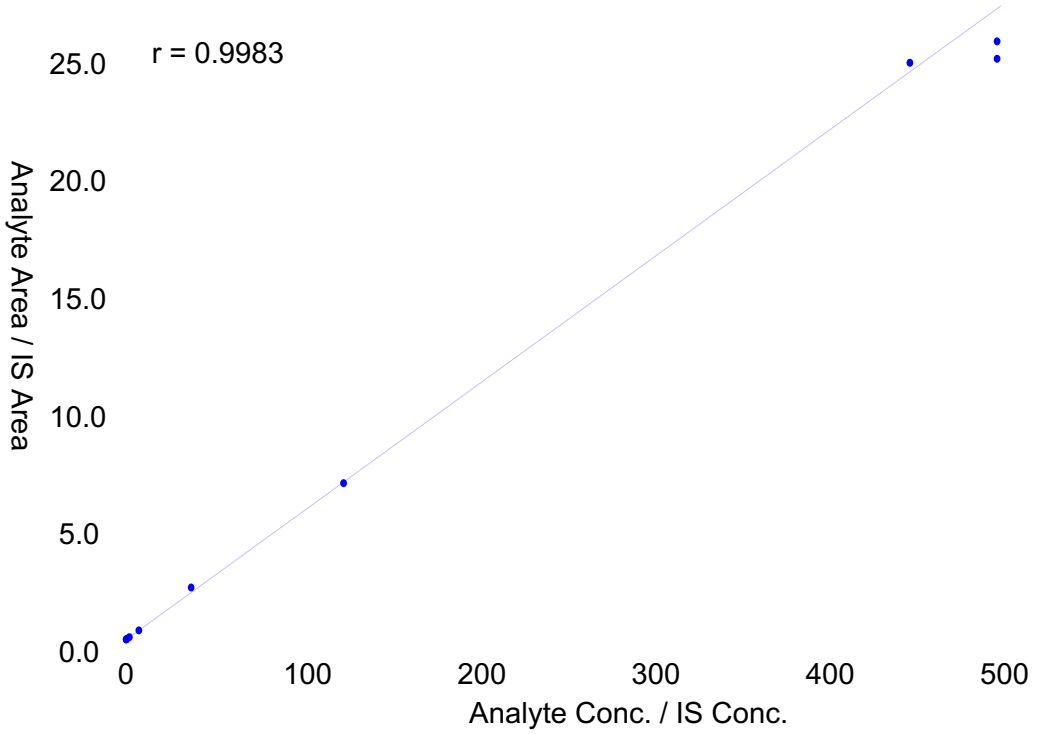


Parent molecule					
n	**18**	**18**	**18**	**18**	**18**
mean	0.0236	0.0745	1.30	25.7	40.3
sd	0.00336	0.00655	0.0532	0.911	1.59
cv [%]	14.2	8.8	4.1	3.5	3.9
accuracy [%]	94.5	99.3	103.6	102.8	100.8

Carboxylic metabolite					
n	**18**	**18**	**18**	**18**	**18**
mean	2.45	7.65	130	2570	3970
sd	0.213	0.298	3.75	88.8	167
cv [%]	8.7	3.9	2.9	3.5	4.2
accuracy [%]	98.0	102.0	103.7	102.9	99.1

Validation results

Linearity and Accuracy and Precision of Qualification samples



Alcohol metabolite					
n	**18**	**18**	**18**	**18**	**18**
mean	0.277	0.760	12.9	253	409
sd	0.0298	0.0709	0.835	18.6	36.2
cv [%]	10.8	9.3	6.5	7.4	8.8
accuracy [%]	110.7	101.4	103.5	101.2	102.3

Challenges during animal study

Sample pre-treatment: 100 µL rat blood + 300 µL acetonitrile

(precipitation directly after sample collection)

Sample storage during Validation and long-term stability test

- PP **safe-lock tubes**

Sample storage of study samples

- PP-tubes (no safe-lock tubes)

Problem: 400 µL sample volume were shipped

Samples with different volumes were arrived

→ PP-tubes were not tight

acetonitrile did evaporate

Conclusion

- We successfully develop and validate two LC-MS/MS methods
- Stability problems were successfully resolved
- Further validations are planned in human plasma and urine.

THANK YOU FOR YOUR ATTENTION!

Any questions?

Feel free to ask!

Acknowledgement

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