

Bioanalysis in practice

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1. Choice of sample preparation method and chromatography

- Sample preparation

Protein precipitation	SPE	Liq/liq
+ Speed + Stability	+ clean-up + sensitivity	+ clean-up + sensitivity
- Sensitivity - clean-up	- stability - costs - clogging	- stability

- Chromatography

- Risks in case of insufficient separation:
 - Isobaric metabolites or labile metabolites (converting to the drug in-source) could co-elute, resulting in overestimation of concentration
 - Other metabolites could co-elute, possibly resulting in ion suppression
 - Chances for co-elution with matrix compounds increases
- Do not sacrifice separation too much for speed. K' at least 3 ?
- Remember: Both good and bad chromatography can be fast

2a) Stability in biological matrices - how to stabilize the unstable ?

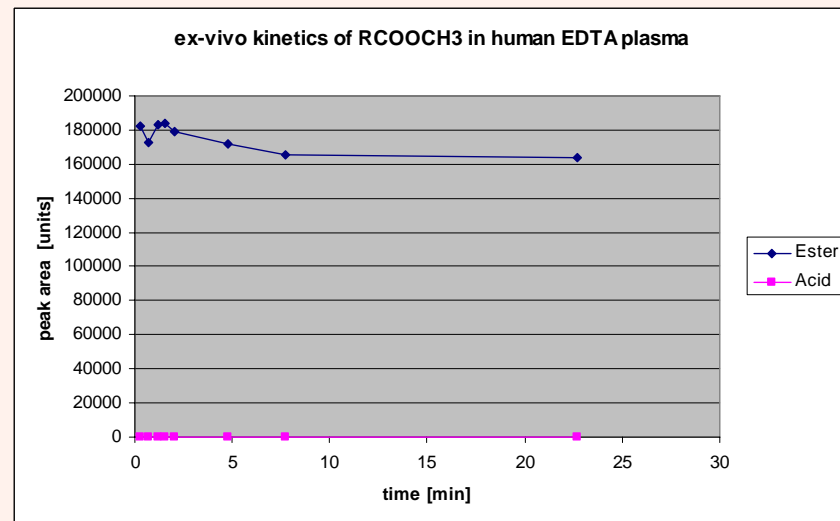
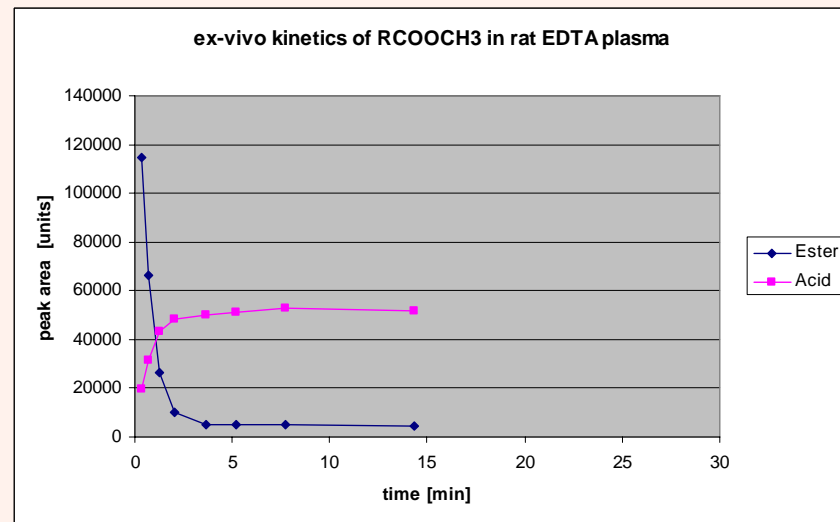
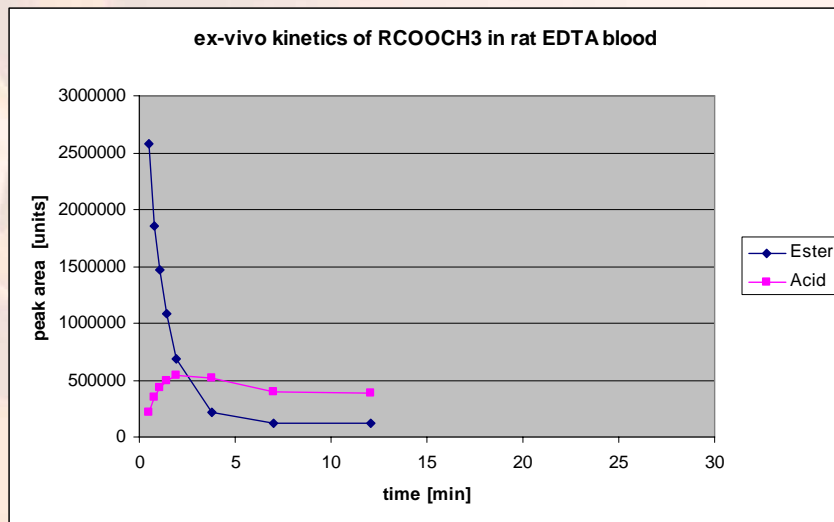
- Various known classes of analytes with potential for instability in biological fluids
 - Esters: Hydrolysis due to presence of esterases in the matrix (most prominent in rodents)
 - Amides: Hydrolysis due to presence of deamidase in the matrix
 - Thiol compounds (Sulfhydryl group): Thiol group is strong nucleophile, may react to form disulfides
 - Compounds sensitive to oxidation may be chemically oxidized in plasma
 - Prodrugs: By definition, these are designed to be instable

- Various possibilities to stabilize the unstable
 - Esterase inhibitors (NaF or phenylmethylsulfonyl fluoride (PMSF))
 - Add anti-oxidant (ascorbic acid, meta bisulfite, EDTA, mercaptoethanol, BHT, ...) --> No single anti oxidant eliminates all kinds of auto-oxidation reactions
 - Derivatize (eg in case of thiols)

Stabilizing Drug Molecules in Biological Samples: J. Chen, Y. Hsieh, Ther. Drug. Monit. 27(2005)617-624

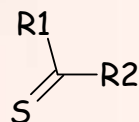
2a) Stability in biological matrices - how to stabilize the unstable ?

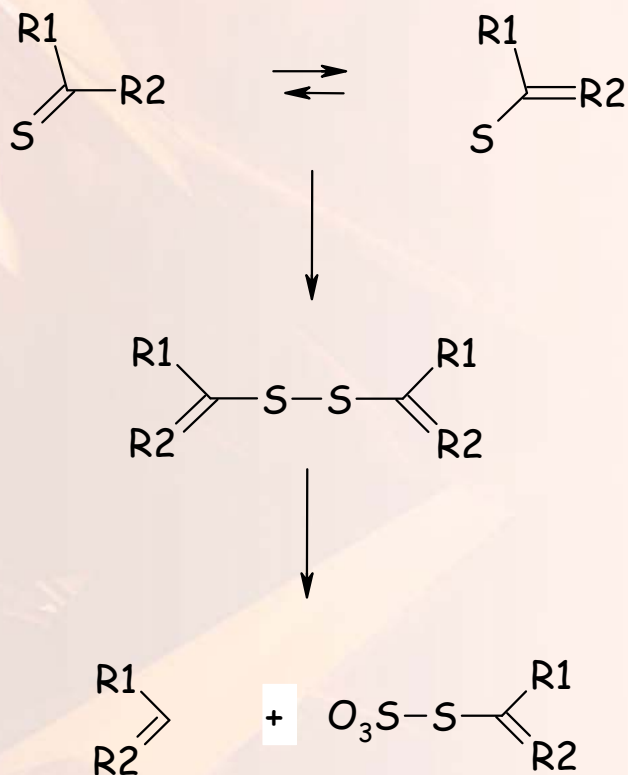
Example 1: Hydrolysis of a methylester in blood and plasma



- $T_{1/2}$ in rat blood and plasma < 2 min
- Stable in non-rodents (dog, rabbit, man)
- NaF, PMSF and paraoxon were not able to stabilize
- Plasma lot-to-lot differences in instability
- Sampling of rodent blood on acetonitrile stabilized the analyte

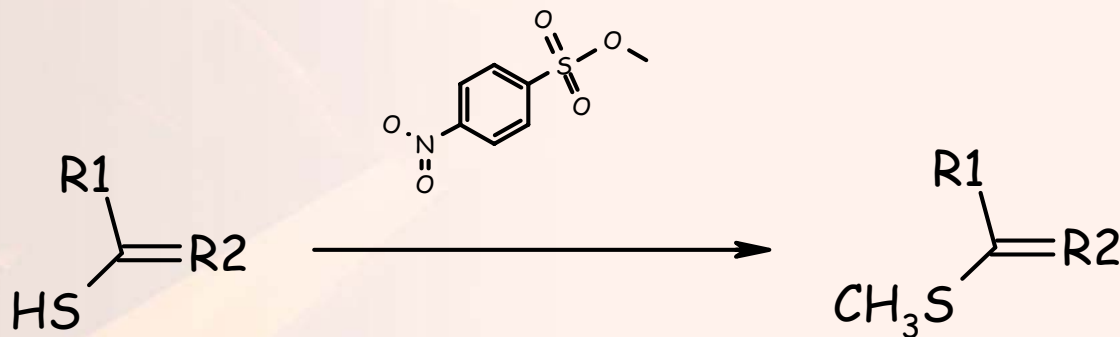
2a) Stability in biological matrices - how to stabilize the unstable ? Example 2: Stabilization of a thiol compound

- Chemical structure: 
- Plasma lot-dependent degradation observed
- Proposed oxidative degradation route:



2a) Stability in biological matrices - how to stabilize the unstable ? Example 2: Stabilization of a thiol compound

- Several stabilization procedures were tested (but did not work adequately)
 - Antioxidants (ascorbic acid and BHT, with and without HCl)
 - collect blood on ethanol, with and without pH adjustment (HCl)
- Final solution:
 - Collect blood on ethanol containing the (stable isotope labeled) IS (to compensate for degradation immediately after sampling)
 - Derivatization by methylation with Methyl Nosylate (MeNOS)

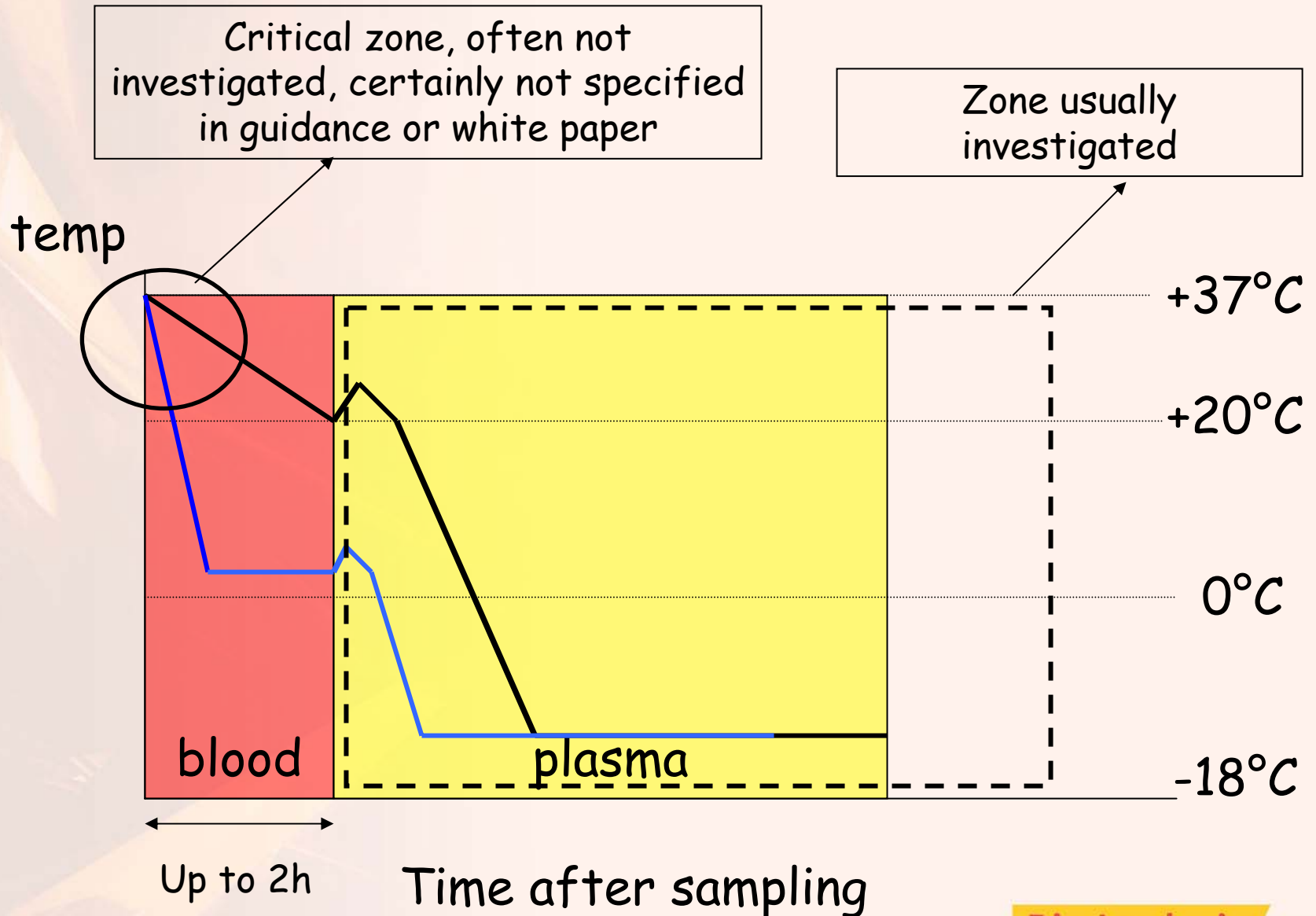


2a) Stability in biological matrices - how to stabilize the unstable ? Example 2: Stabilization of a thiol compound

(All sample handling in the dark and under yellow light conditions)

- Add 2 ml of a solution of internal standard (SIL) in ethanol to a Polypropylene tube, weigh before and after addition.
- Collect 300 μl of blood directly into the tube.
- Vortex for **10 seconds**
- Weigh the tube again
- Centrifuge at 1900 x g for 10 min **ASAP** after addition of blood to the ethanol.
- Transfer 500 μl of the supernatant to a brown polypropylene tube. TAKE CARE: leave the pellet intact!
- Add immediately (within 30 minutes after blood sampling) 25 μl 5 mg/100 μl MeNOS in DMF.
- Incubate at room temperature while continue vortex mixing for 15 min
- Add 500 μl 0.01 M ammonium formate buffer (pH 4 met formic acid)
- Vortex mix briefly
- Store the sample in the freezer until analysis

2b) Stability: Blood stability issues



2b) Stability: Blood stability issues

Consider difference:

- enzymatic reactions - chemical reactions vis-à-vis temperature changes
- Time spent in blood at critical temperature
- Time spent in plasma at non critical temperature

In general :

- Compounds instable in plasma are always unstable in blood
- Compounds stable in plasma are not necessarily stable in blood
- Blood stability experiments (in fresh whole blood) are both tedious and difficult to interpret

2c) Stability: Labile metabolites

- Various known classes of metabolites with potential for instability in biological fluids
 - N-oxides: can convert back to parent drug under various conditions:
 - In the sample (blood bigger issue than plasma)
 - During extraction (especially under alkaline conditions and during dry-down step)
 - By exposure to light
 - In the mass spec interface (risk in case of co-elution)
 - Note 1: Also N-oxides can be formed during sample prep (dry down step is a risk)
 - Note 2: use of ascorbic acid to stabilize parent will also lead to reduction of N-oxide metabolites !

Labile metabolites: D. Dell, Chromatographia Suppl. 59(2004)S139-S148

2c) Stability: Labile metabolites

- Various known classes of metabolites with potential for instability in biological fluids
 - Glucuronide conjugates:
 - Ester-glucuronides (acyl-glucuronides):
 - » Hydrolysed @ acid and basic pH and in presence of esterases (compound dependent)
 - Ether-glucuronides:
 - » Usually somewhat more stable than ester glucuronides
 - *N*-glucuronides (of prim., sec. And tert N)
 - » Hydrolysed @ acid and basic pH and @ high temp (compound dependent)
 - As with *N*-oxides, conversion to parent in sample, during extraction and in the mass spec interface can occur
 - Diastereomers (sometimes, depending on chemical structure)

Labile metabolites: D. Dell, Chromatographia Suppl. 59(2004)S139-S148

2c) Stability: Labile metabolites

Example 1: Strategy for optimization of method for labile *N*-oxides

- Search for potential *N*-oxides in study samples
- Confirm identity using MS-spectra (may not always be possible)
- Isolate metabolite(s) by HPLC - fraction collector
- Check stability by spiking isolated metabolite(s) to blood and plasma
 - Check for appearance of parent after sample prep.
 - If needed, optimize sample preparation using isolated metabolite(s) (until no degradation to parent during sample preparation occurs)
- Evaluate stability of parent AND isolated metabolite(s) in blood and plasma to determine final sampling conditions

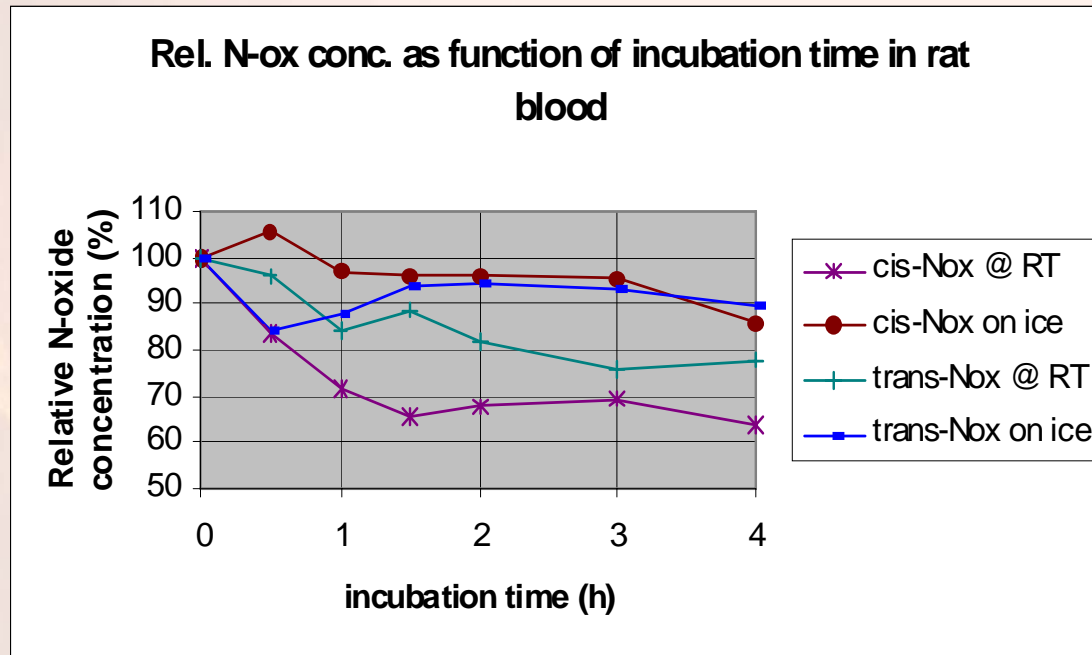
2c) Stability: Labile metabolites

Example 1: Strategy for optimization of method for labile *N*-oxides

- Instability of *N*-oxides often more apparent in blood. No reference compounds required for strategy, so generally applicable
- Enhanced Bioanalytical strategy gives greater confidence in correctness of results
 - Optimized sample preparation
 - Optimized sampling conditions
- Strategy reduces the chance that incurred stability and reproducibility tests will fail
- Same strategy can be applied for all other metabolites that can possibly convert back into parent

2c) Stability: Labile metabolites

Example 1: Strategy for optimization of method for labile *N*-oxides



- Typical result for one of our drugs in development (note the presence of both cis- and trans-isomers of *N*-oxide) - see above
- Sampling of blood on ice and centrifugation within 1 hour (preferably within 30 min after sampling to be on safe side)
- No degradation of *N*-oxides observed after 2 hours at RT in plasma

2c) Stability: Labile metabolites

Example 2: Ester glucuronide

- Drug in development, chemical structure RCOOH
- Experiments conducted to assess stability of metabolite RCOO-Gluc in human blood, plasma and urine in order to advice on sampling conditions:
 - Rat dosed with RCOOH
 - Bile collected (containing both RCOOH and RCOO-Gluc) and spiked into blood, plasma and urine (@ different pH's)

2c) Stability: Labile metabolites

Example 2: Ester glucuronide

Matrix	Incubation conditions	Remarks	% of reference unchanged drug	% of reference Acylgluc (sum of isomers)
Plasma	1 hours at 0°C (ice) 4 hours at RT		97 287	88 41
Blood	30 min at 0°C (ice) 4 hours at RT	plasma analysed	99 248	97 45
Blood	30 min at 0°C (ice) 4 hours at RT	whole blood analysed	114 300	98 35

pH urine	Incubation conditions	% of reference unchanged drug	% of reference Acylgluc (sum of isomers)
2.8	4 hours at 4°C	95.2	96.9
	12 hours at 4°C	110	105
4.3	4 hours at 4°C	105	105
	12 hours at 4°C	108	96.0
5.8	4 hours at 4°C	110	106
	12 hours at 4°C	113	90.4
7.9	4 hours at 4°C	253	54.8
	12 hours at 4°C	349	25.8

- Acyl-glucuronide is stable in human heparin plasma for 1 hour on melting ice, but NOT for 4 hours at RT
- Acyl-glucuronide is stable in human heparin blood for 30 min on melting ice, but NOT for 4 hours at RT.
- Acyl-glucuronide is stable in human urine in pH range 2.8 - 5.8 up to 12 hours at 4 degrees C, but NOT stable at higher pH (at pH 7.9 50% degradation after 4 hours @ 4 degrees C)

2d) Stability: Stability to light

- Can be a difficult parameter to test as there exist different 'light' conditions
 - Miami, Florida on a sunny summer day outside
 - Belgium sunny summer day in the lab, near the window
 - Belgium sunny summer day in the lab, not near the window
 - Belgium rainy autumn day...
 - Etc
- A light cabinet (e.g. Hereaus Suntest CPS+) can be used to create standard conditions



2d) Stability: Stability to light

- Test to correlate Suntest CPS+ with lab conditions shows that 3 hours in suntest correlates well with 3 days under 'worse case' lab conditions

<u>solvent</u>	<u>incubation conditions</u>	<u>compound 1</u>	<u>compound 2</u>	<u>compound 3</u>	<u>compound 4</u>	<u>compound 5</u>
methanol	0.5 h Suntest	99	72	63	82	60
methanol	1 h Suntest	98	52	42	79	40
methanol	3 h Suntest	95	14	17	65	5
methanol	8.5 h Suntest	100	0	12	73	1
methanol	3 d yellow light	98	95	94	95	95
methanol	3 d fume hood	94	100	98	103	96
methanol	3 d sunlight behind glass	98	5	2	79	1
DMSO	0.5 h Suntest	100	54	78	66	53
DMSO	1 h Suntest	101	26	52	70	28
DMSO	3 h Suntest	99	9	13	58	7
DMSO	8.5 h Suntest	98	2	2	55	1
DMSO	3 d yellow light	102	109	101	101	95
DMSO	3 d fume hood	101	109	100	95	95
DMSO	3 d sunlight behind glass	94	5	2	60	1

- Challenge: Sampling under 'yellow light' feasible in pre-clinical, more difficult in phase 1 trials and 'impossible' in phase 3 --> 'better safe than sorry' works fine for preclinical, but more thorough investigation needed for clinical to avoid sampling under yellow light if possible

3) Adsorption

- Elimination of adsorption effects is important in low protein containing matrices :
 - Plasma protein binding studies
 - CSF samples
 - Urine samples
 - Caco-2 and other transporter studies

- Or during some steps of sample prep :
 - Very clean L/L extracts
 - Extreme dilutions in API-5000 assays

3a) Adsorption:

Example 1: Adsorption of analyte from working solutions

Analyte standard [ng/ml]	preparation in methanol		preparation in plasma	
	% deviation	relative response	% deviation	relative response
1	7.7	74	3.5	102
2	-10.6	63	1.5	100
5	-3.7	72	-1.3	97
10	7.3	84	-1.6	96
20	-1.3	80	1.1	98
50	2.3	88	-1.4	96
100	0.8	90	-11.0	86
200	2.1	95	4.9	101
500	-1.1	97	0.7	97
1000	-2.2	100	4.5	100

$$\log y = 1.06 \log x + 0.0351 \quad \log y = 0.995 \log x + 0.194$$

the relative response = normalized peak area/concentration ratio

3b) Adsorption:

Example 2: Adsorption of analyte from low protein matrix (urine)

- In our lab, adsorption potential of analyte from urine matrix is routinely tested prior to start of the FIH trial
- Set-up of the experiment
 - Collect urine and bring to pH relevant for real-life situation (we test 4.5 - 6 - 8)
 - Transfer 100 ml to 1 l collection container (PE or glass) both with and without additives (2% BSA and 0.2% tween 80) and spike with compound @ 10x LLOQ
 - Store for 24 hours at room temperature
 - Transfer to new recipient of same material twice (to simulate volume measurement)
 - Store sample in freezer at least overnight until analysis
 - Compare with reference sample which is directly spiked before analysis in same matrix, pH and with same additive

pH	4.5		6		8	
Recipient additives	Glas	PE	Glas	PE	Glas	PE
No additives	90	82	86	54	75	25
2 % BSA	101	100	99	94	94	85
0.2 % TWEEN-80	105	101	106	99	100	101

3b) Adsorption:

Example 2: Adsorption of analyte from low protein matrix (urine)

- Point of concern for the analysis: Assure that no ion suppression is caused by the tween 80
- In case of ion suppression, SPE (mixed mode cation exchange) or liq/liq extraction (with sufficiently apolar solvent) are good options to remove the tween 80

Extraction method	%tween-80 (marker peak)
SPE on Oasis HLB, wash with 5% methanol	100
SPE on Oasis HLB, wash with 20% methanol	91
SPE on Oasis HLB, wash with 50% methanol	92
SPE on mixed mode cation exchange (Certify)	6
L/L extraction @ pH 9 (borax) with 95/5 heptane/iAA	0.2
L/L extraction @ pH 9 (borax) with 90/10 heptane/iAA	0.8
L/L extraction @ pH 9 (borax) with EtOAc	87

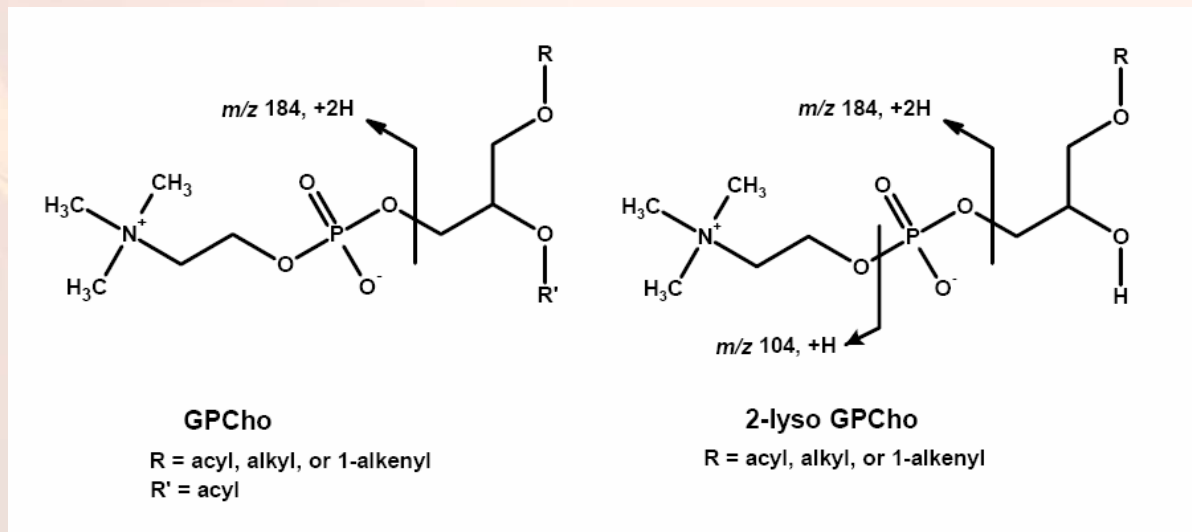
4) Ion suppression / enhancement:

General

- Guidance states % CV over 6 lots of matrix should be <15%
- Also, absolute matrix effect (Matrix Factor, MF) is mentioned but no criteria
- In case of matrix effect, several ways to reduce / eliminate, such as:
 - Post-column infusion experiments to localize suppression area to optimize chromatography
 - Optimize LC
 - Optimize extraction
 - Etc
 - See literature

4a) Ion suppression / enhancement: Phospholipids

- Phospholipids identified as a major source of matrix effects in plasma (but, as always, compound dependent !)
- Glycerolphosphocholines (GPCho's) most abundant, but there are others too !



- GPCho's can be monitored by IS-MRM (monitoring of 184 → 184 and 104 → 104 @ high DP and low CE)
- Can either be removed by extraction or by special HPLC conditions

J. L. Little, M. F. Wempe, C. M. Buchanan, *Journal of Chromatography B*, 833 (2006) 219-230.

http://littledomain.com/james/files/matrix_effects.htm

4a) Ion suppression / enhancement: Phospholipids

Removal of phospholipids by chromatography

- Step gradient to high organic (98-100%) required (use MeOH rather than ACN)
- High column temperature beneficial for phospholipid removal
- pH has no influence
- To further speed up analyses, a shorter column and a higher flowrate during wash step can be applied

J. L. Little, M. F. Wempe, C. M. Buchanan, *Journal of Chromatography B*, 833 (2006) 219-230.

http://littledomain.com/james/files/matrix_effects.htm

4a) Ion suppression / enhancement: Phospholipids

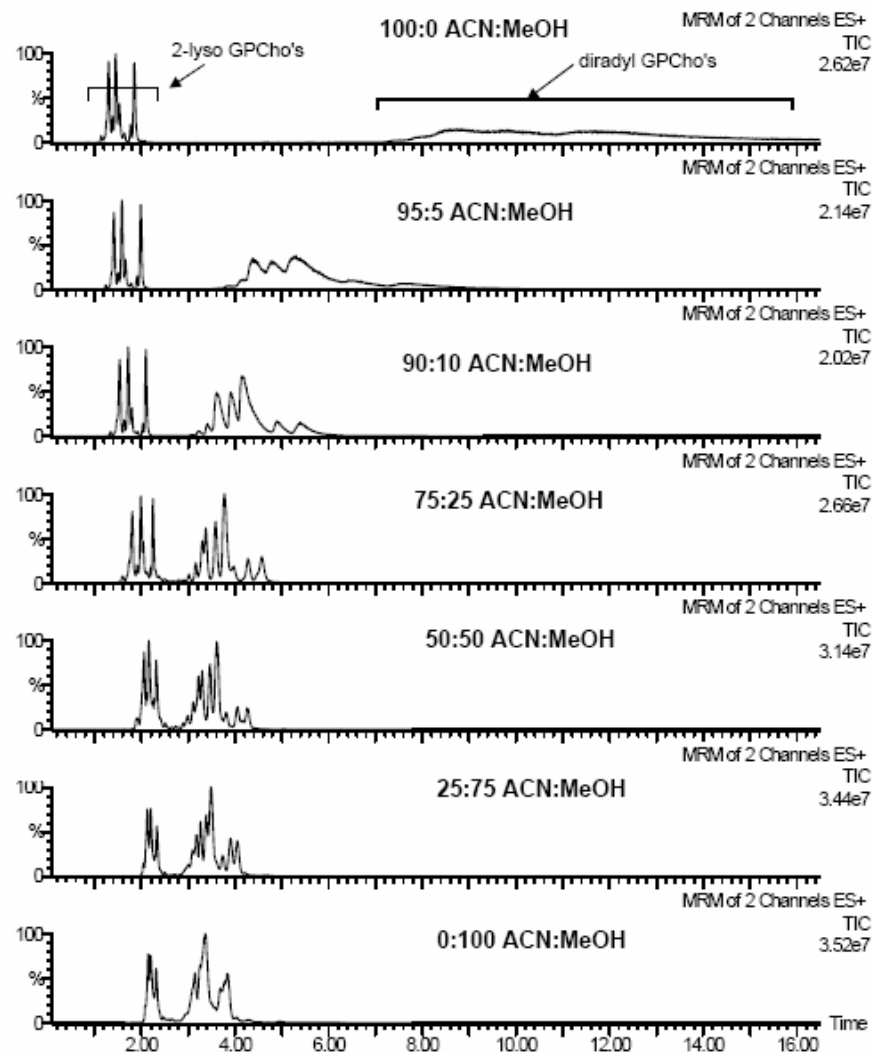


Fig. 5. Comparison of solvent composition for the elution of GPCho's from reverse phase HPLC column employing the same gradient elution for all separations utilizing MeOH and ACN as the organic eluents.

4a) Ion suppression / enhancement: Phospholipids

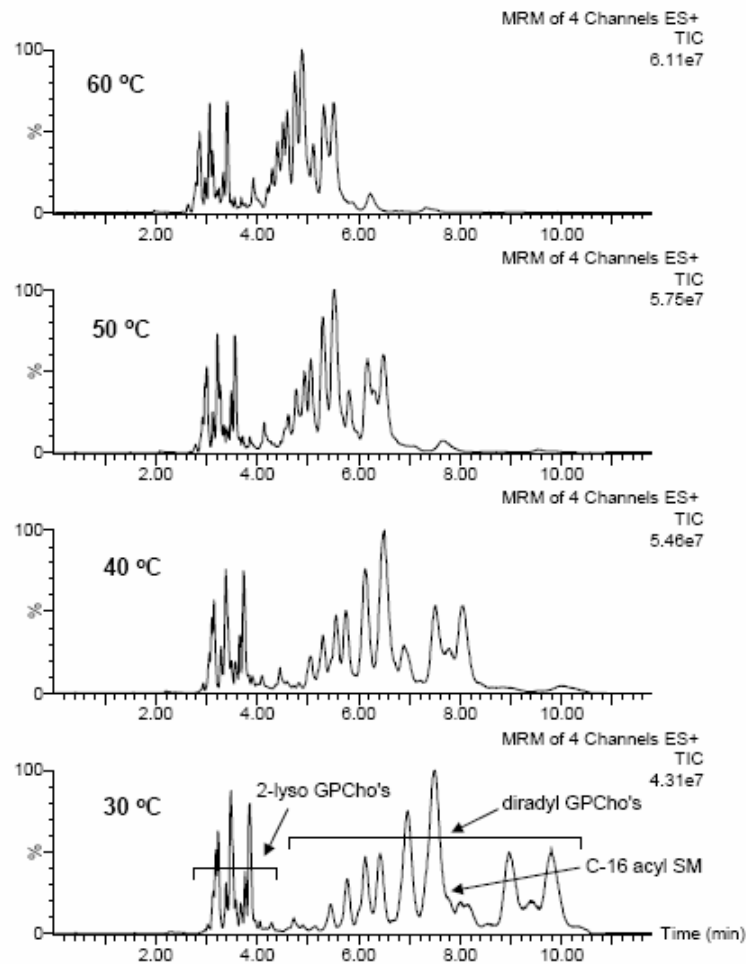


Fig. 6. Comparison of column temperature on the elution of GPCho's from reverse phase HPLC column employing the same gradient for all separations utilizing 50:50 mixture of MeOH:ACN for Solvent B and ammonium formate buffer at pH 3.5 for solvent A.

4a) Ion suppression / enhancement: Phospholipids

Example: Optimize HPLC conditions to remove phospholipids

Method 1

- Sample prep: Liq/liq extraction @ basic pH with TMBE
- Column: 50 x 4.6 mm (3.5 um) Symmetry Shield RP18 @ RT
- Mobile phase:

Time [min]	Flow [ml/min]	0.1% formic acid [%]	Acetonitrile [%]
0.00	1.2	25	75
2.50	1.2	25	75

Method 2

- Sample prep: Liq/liq extraction @ basic pH with TMBE
- Column: 30 x 4.6 mm (3.5 um) Xbridge C18 @ 40 degrees
- Mobile phase:

Time [min]	Flow [ml/min]	0.1% formic acid [%]	Methanol [%]
0.00	1.5	22	78
0.75	1.5	22	78
0.80	3	2	98
2.30	3	2	98
2.35	1.5	22	78
3.00	1.5	22	78

HPLC method	%CV of Peak Area Internal Standard (IS)	%CV of Peak Area ratio Analyte / IS
Method 1	44.7% (n=145)	5.4% (n=145)
Method 2	4.5% (n=187)	1.7% (n=187)

4a) Ion suppression / enhancement: Phospholipids

Removal of phospholipids by extraction

- E. Chambers et al., J. Chromatogr. B 852(2007)22-34 (Waters)
 - SPE removes part of phospholipids, MCX-SPE most powerful
 - Liq/liq extraction with MTBE @ neutral or basic pH comparable with MCX-SPE (MeOH/NH₄OH elution) in phospholipid removal
 - Elution of MCX-SPE with acetonitril/NH₄OH instead of MeOH/NH₄OH further reduces phospholipids by factor 20.
- Lanthanide sorbent column has been developed by Tandemlabs to remove phospholipids

4b) Ion suppression / enhancement: By formulations

- Prerequisites for a formulation to cause a potential problem:
 - Presence in plasma (so after PO dosing only if Mwt < 500 Da)
 - Co-elution with analyte (polar formulations often not a problem)
- Known formulations that could cause problems (according to literature):
 - Polysorbate 80 (tween 80)
 - PEG 400
 - Hydroxypropyl- β -cyclodextrin

W. Shou, N. Weng, Rapid Comm. Mass Spectrom. 17(2003)589-597

X. Xu et al., Rapid Comm. Mass Spectrom. 19(2005)2643-2650

P.J. Larger et al., Journal of Pharm. And Biomed. An. 39 (2006) 206-216

4b) Ion suppression / enhancement: By formulations In-house obtained results

- Removal of formulations by extraction:

Extraction method	%tween-80 (marker peak)	%OH-prop-B-CD	%PEG-400 (marker peak)
SPE on Oasis HLB, wash with 5% methanol	100	100	100
SPE on Oasis HLB, wash with 20% methanol	91	90	89
SPE on Oasis HLB, wash with 50% methanol	92	2	5
SPE on mixed mode cation exchange (Certify)	6	1	3
L/L extraction @ pH 9 (borax) with 95/5 heptane/iAA	0.2	0	0.1
L/L extraction @ pH 9 (borax) with 90/10 heptane/iAA	0.8	0	0.1
L/L extraction @ pH 9 (borax) with EtOAc	87	0	4

- PK of formulations after IV dosing:

	tween-80	OH-prop-B-CD
t _{1/2} [min]	60	45
Relative conc. @ T=2 hrs (rel to T=2 min) [%]	10	8
Relative conc. @ T=6 hrs (rel to T=2 min) [%]	1	-

- Ways to test influence on results:
 - Standard addition to incurred sample
 - Analyse undiluted and after 10x dilution (GSP = always analyse time points up to T=30 min after IV dosing after 10x dilution to prevent problems)

4b) Ion suppression / enhancement: By formulations In-house obtained results

- Chromatography of formulations: - Mass transitions

- Column: Hypersil BDS C18
50x4.6 mm, 3 μ m
- Mobile phase:

Time [min]	Flow [ml/min]	0.01 M AmFa pH 4 [%]	ACN [%]
0.00	1	90	10
5.00	1	10	90
6.00	1	10	90
6.10	1	90	10
8.10	1	90	10

- Retention times of formulations:

Analyte	retention time(s) [min]
OH-prop-B-CD	1.0 - 2.0
tween-80	2.0 - 3.0; 5.5 - 7.5
PEG-400	1.2 - 3.0

Analyte	mother	daughter	DP	FP	CE
OH-prop-B-CD	643.6	221.2	31	170	29
	701.6	221.2	31	170	29
	759.7	221.2	31	170	29
	817.6	221.2	31	170	29
tween-80	586.8	309.4	51	200	31
	661.6	309.4	51	200	31
	826.8	309.4	51	200	31
PEG-400	300.3	133.1	21	120	23
	432.4	133.1	36	140	29
	652.6	133.1	46	220	37

4c) Ion suppression / enhancement: By container materials

- An example from a recent clinical study
 - Samples from a phase 1 clinical study were analysed using an LC-MS/MS method with an analogue as IS.
 - IS Peak area was significantly lower in cal curve and QC samples than in study samples
 - Blood collection procedure for the blank plasma for preparation of cal's and QCs used plastic 'blood-bank type' bags. Probably, a plasticizer from the bags (di-octyl phthalate ?) caused suppression
 - Problem solved by using plasma obtained using vacutainer tubes.

4d) Ion suppression / enhancement:

By metabolites and by (metabolites of) co-medications

Proposed procedure for assessment

- metabolites of analyte
 - Check if no co-elution with major metabolites
 - In case of co-elution check for suppression by spiking both metabolite and drug OR by doing standard addition experiments
- (metabolites of) co-medications
 - Check if no co-elution with (metabolites of) co-medications (use incurred samples if no ref compound is available)
 - In case of co-elution check for suppression by spiking both (metabolite of) co-medications and drug OR by doing standard addition experiments

5) What about matrix lot-to-lot differences...?

- Influence of different types of plasma on matrix effects and stability?
 - Pre-clinical
 - Composition plasma relatively constant because of specific strain used
 - Still variations possible (fasted/fed, type of food, healthy/sick, age)
 - Clinical
 - Fasted / fed
 - Young / elderly
 - Caucasian / non-caucasian
 - Poor / extensive metabolisers
 - Healthy / sick
 - Hepatically impaired / renally impaired
 - Lipemic
 - Etc.
- Influence of age of the plasma on matrix effects and stability?
 - Anti-oxidants (eg ascorbic acid) degrade with time
 - pH of plasma raises upon storage due to CO₂ exchange
(A. Fura et al., Journal of Pharm. And Biomed. An. 32 (2003) 513 - 522)
- Need good scientific judgement in order not to miss relevant issues

6) Conclusion

- Bioanalysis in practice is more than following a Guidance / Guideline and some SOPs
- A good assay is more than standards and QCs pass
- Thorough method development and GSP (good scientific practice) are of key importance
- For development of an optimal Bioanalytical method, various aspects (such as physicochemistry, biochemistry, metabolism etc) should be taken into account



