Stability Issues in Bioanalysis: New Case Studies

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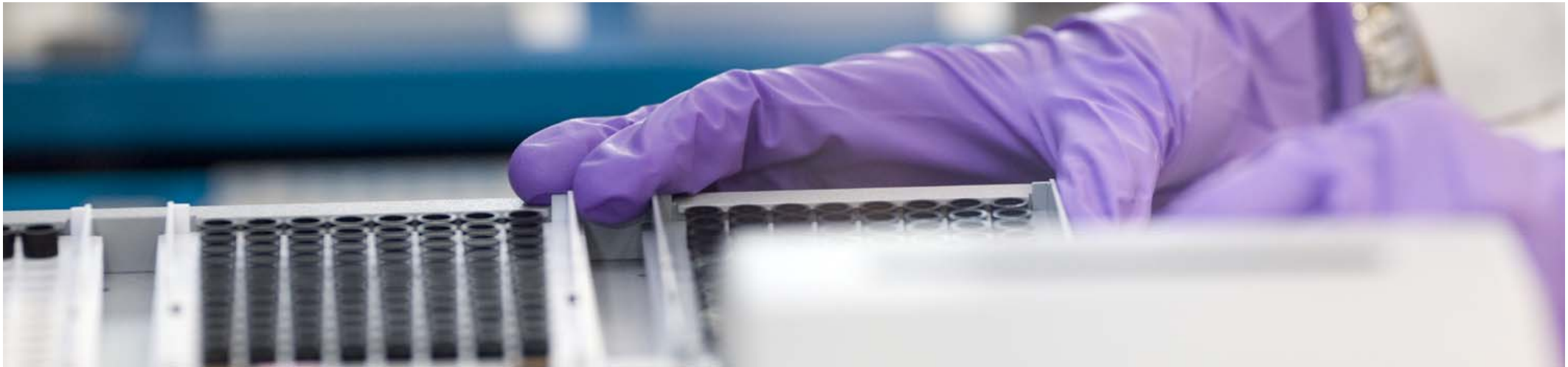

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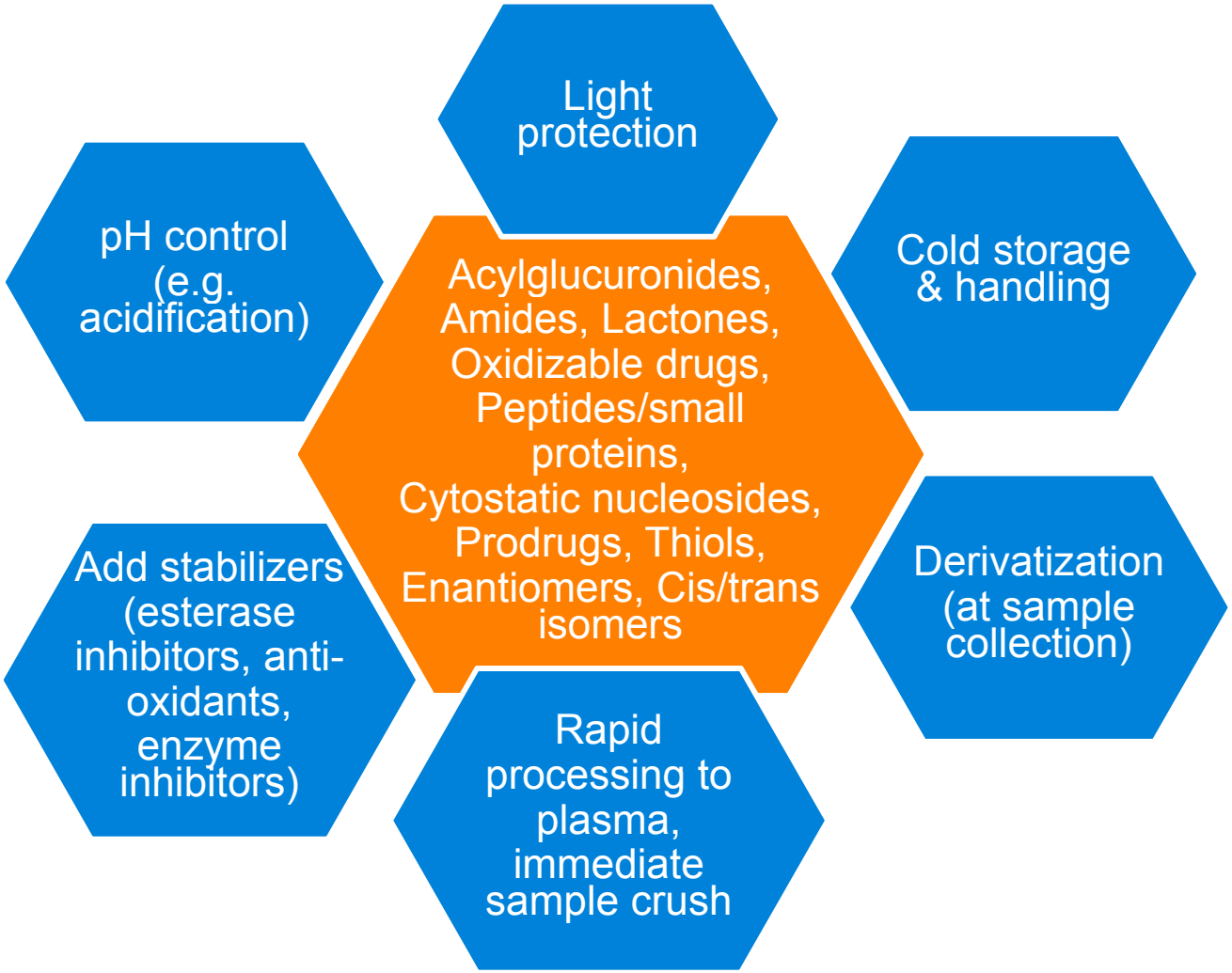
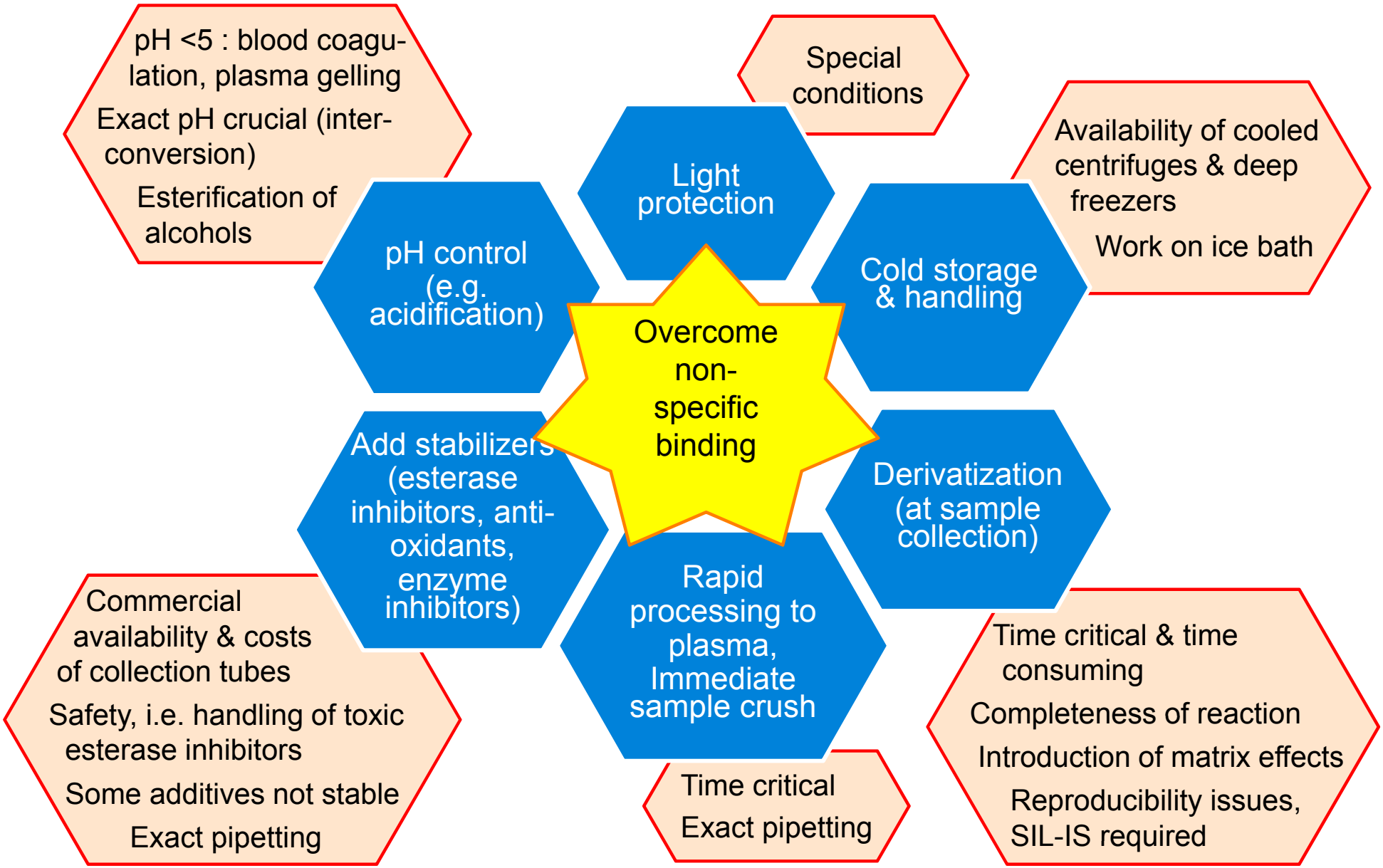
Overcoming stability issues

- Analyte stability must be ensured during **sample collection, processing, storage, extraction and duration of analysis** to generate reliable bioanalytical data.

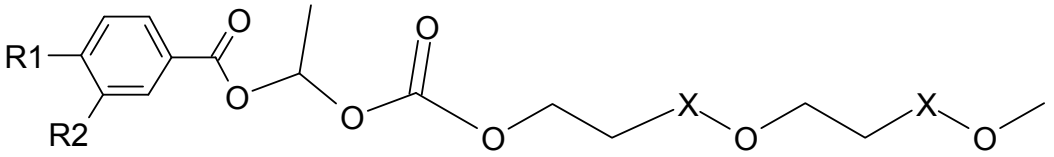


Overcoming stability issues – an easy task?



Pegylated prodrug – preclinical method

Ester prodrug pegylated at benzoic acid moiety
→ **Active drug** & **total drug** (prodrug + active) measured



polydispersed polymer with median MW ~2600

Stabilize prodrug with esterase inhibitor di-isopropyl fluorophosphate (DFP) and **measure "free" active drug**

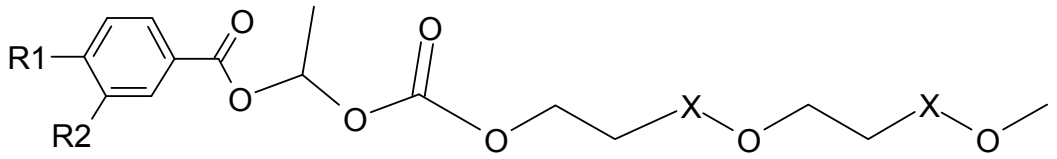
Plasma	Collect in K3-EDTA tubes pre-added with 10 µL of 5% DFP (aqueous solution) per 0.5 mL blood (final conc. 0.1% DFP in blood). Mix gently by inversion. Put on ice, centrifuge within 30 min of collection. Store plasma at -70°C.
CSF	Collect into tubes. Measure CSF volume, add 10 µL 5% DFP per 0.5 mL CSF.
Brain	Collect & flash freeze as whole, weigh frozen, keep on dry ice after collection, store at -70°C. Homogenize in solvent containing 0.1% DFP.

Hydrolyze prodrug with 0.1 N NaOH and **measure "total "active drug**

Calculate prodrug concentration: difference total – free active drug

Pegylated prodrug – clinical method

Active «free» and «total» measured



➢ **Di-isopropyl fluorophosphate (DFP) not well suited in clinic!**

CAUTION!

DFP is **very toxic**; avoid skin contact, breathing vapors, etc.! The preparation of solution should be performed under safe conditions (fume cabinet etc.).
DFP is a **very unstable** compound; 5% DFP solution in water should be freshly prepared, just before samples collection and kept on ice!

➢ **Dichlorvos used!**

- Less potent → less toxic
- More stable (40% solution in water/methanol kept 7 days at -20°C)

Sample processing (**all samples kept on wet ice**)

→ Centrifuge at 4°C within 30 min →

Whole blood collected in K3-EDTA tube

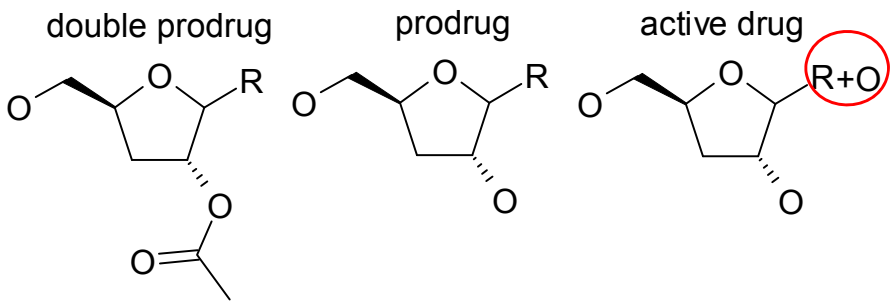
Plasma tube pre-spiked with dichlorvos (5µL/mL)

Stable in whole blood during processing - stabilizer added to plasma tube only

Acetic acid ester double prodrug – clinical method

Double prodrug needs stabilization in plasma

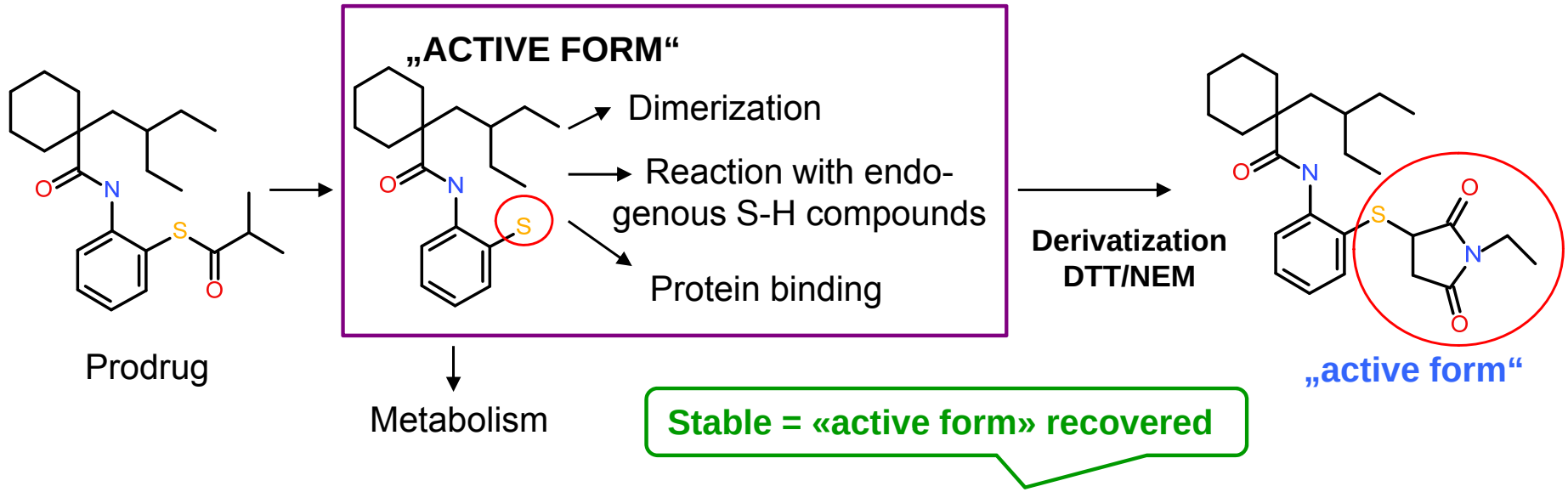
Analysis of
- double prodrug
- prodrug
- active drug
- metabolite (tetrahydrofurane ring cleavage)



	Towards a practical solution
1. Test of different esterase inhibitors to stabilize	• NaF, phenylmethane-sulfonylfluoride, bis(4-nitrophenyl)-phosphate, eserine, acetylcholine, dichlorvos (0.1, 1 & 10 mM tested) ➢ Some inhibitors are toxic → not well suited for a clinical setting
2. Select most suited inhibitor for clinics (non toxic)	• Samples collected in tubes containing K3-EDTA, 10 mM NaF (10 µL 1M NaF in water per 1 mL blood) and 10 mM citric acid (10 µL 1M citric acid in water/1 mL blood); kept on ice until centrifugation ➢ Not toxic but customized tubes preparation needed
3. Clinics requested test of commercial tubes	• Samples collected in BD Vacutainer® Plus plastic sterile tubes containing 15 mg NaF and 12 mg potassium oxalate & kept on ice until centrifugation ***(NaF is an esterase inhibitor and the K-oxalate acidifies = we use both mechanisms of stabilization)*** ➢ Stabilization successful

→ **Start with simplest option (commercial tubes suitable?)**

Thioester prodrug – dalcetrapib



- Active compound not available as chemical entity to test stability
- Thioester for stability: spike, store, hydrolyze, derivatize - ***does not reflect real samples!***

Stability QCs (thioester prodrug spiked)	Incurred samples stability
• Light sensitive • Not stable in EDTA but heparin plasma • Not stable in «old» EDTA & heparin plasma (collected >1 month before use) • Not stable in rodent plasma (non-ester cleavage related instability)	• Not particularly light sensitive • Stable in EDTA & heparin • Stable in «old» plasma (human heparin 4 months -20°C tested) • Stable in rat & mouse plasma (18h RT & 6 weeks -20°C)

→ **Incurred samples stability (ISS) can be alternative; part of ISR or separately**

Peptide challenges: GLP-1 analogues

- Taspoglutide - natural GLP-1 but 2 AA exchanged with aminoisobutyric acid (Aib)
- Fatty acid substituted GLP-1 analogs (with and without Aib)

Strong non-specific binding

- Apparent instability in neat solution (adsorption effects, low concentrations)
- Substance loss during sample preparation
- Carry-over in the LC-MS/MS system

➢ **>30% organic solvent** in solutions & mobile phases to keep peptide in solution

➢ **Avoid evaporation and reconstitution** steps to prevent compound loss

➢ Highly protein bound → **well stabilized in plasma**: prepare “stock solutions” in plasma

Potential cleavage by dipeptidylpeptidase IV (DPP IV)

- N-terminal cleavage, particularly if proline or alanine in 2nd position of sequence

➢ **Precautionary addition of protease inhibitor**

Plasma samples collected in **K3-EDTA** tubes, **50 KIU aprotinin/mL blood** is added, kept on ice until centrifugation; 100 KIU aprotinin / mL of plasma for preparation of CALs/QCs

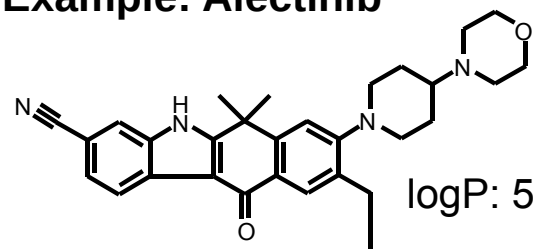
Preventing non-specific binding in urine

pH control | additives | low bind material

These situations can occur when adding stabilizers:

Compound sticks to container but can easily be desorbed

Example: Alectinib

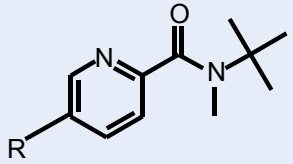


logP: 5.8

- Add an amount (e.g., 1%) of required solution (containing Tween or other agent) **after collection** of all periods of a certain time interval (e.g., 0-24 h).
- Requires weighing urine bottle / measuring urine volume.
- Common procedure, works for most «sticky» analytes.
- Usually, 0.1 to 0.2% Tween (20 or 80) is sufficient.

Compound is very unstable or sticks irreversibly to the container

Example: drug & desmethyl metabolite



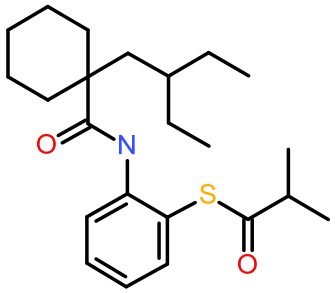
logP: 3.9

- Agent needs to be added to collection bottle **before urine is collected**; volunteer urinates on top of the stabilizer.
- Required percentage of additive is known but not the amount of urine that will be produced.

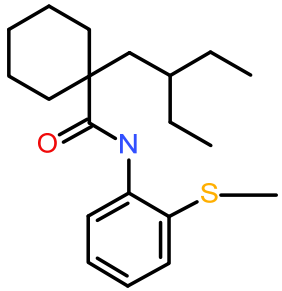
➢ **Validate min/max concentration of additive (e.g., 0.1-1% Tween 80), all percentages in between are considered covered.**

Method transfer issues for urine

1) Dalcetrapib (lipophilic drug) and metabolite



logP: 6.8



logP: 6.2

➢ **Method development :**

- no stabilizing agent needed
- no special precautions

➢ **Method validation :**

☹ **Exponential calibration curves**

= concentration dependent non-specific adsorption

(no adsorption only in urine from donors used during method development)

☺ **Clinical study not started**

The following instructions were described in lab manual and followed:

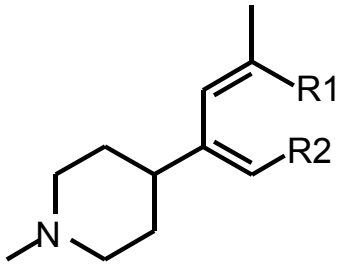
→ Add 0.1% **Tween 80** at urine collection (CALs & QCs prepared in urine w. Tween)

→ Use **glass containers** for urine collection, storage and sample preparation

→ **Investigate adsorption early in method development using multiple urine donors**

Method transfer issues for urine

2) Basic drug and desmethyl-metabolite



logP: 3.8, 3.6

➢ **Method development :**

- no stabilizing agent needed
- storage tubes (low bind) should be almost full to minimize surface/liquid ratio
- sonication of samples (for at least 10 minutes) that were frozen to homogenize

➢ **Method validation :**

☹ **>50% of LLOQs & LQCs failed (negative bias)**

= **nonspecific adsorption in processed samples suspected** (injection solution)

☹ **Clinical study samples were already taken!**

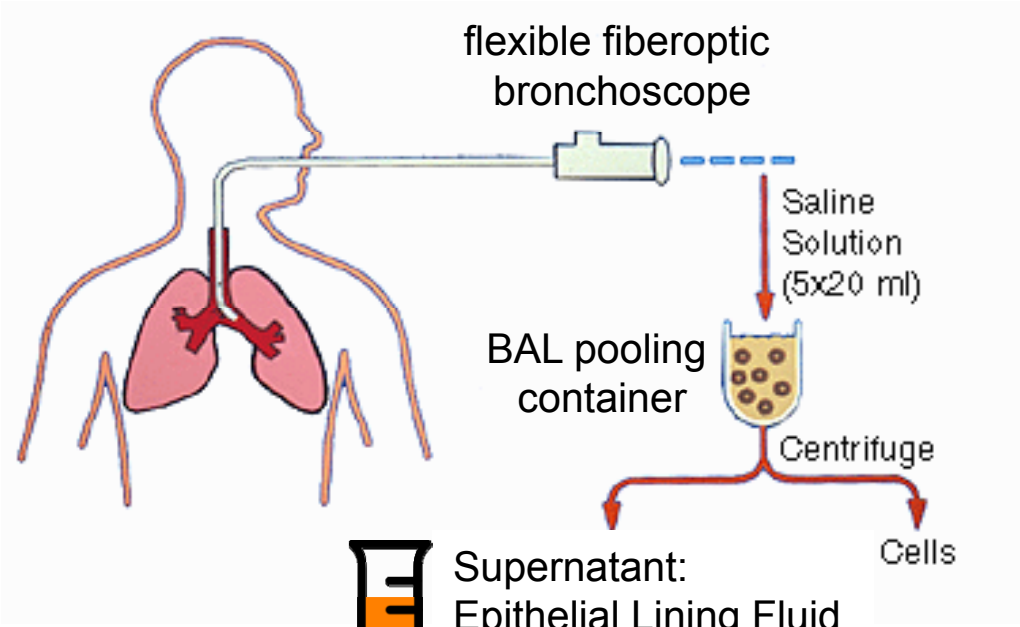
→ Addition of BSA to blank urine used to prepare CALs & QCs

→ Addition of BSA to every clinical urine sample once received at Lab

→ Stability tests in urine with and without BSA (to cover storage of clinical samples before BSA addition in the BA Lab)

→ **Consider testing of all sample handling & preparation steps**

Bronchoalveolar Lavage (BAL) – Non-specific binding



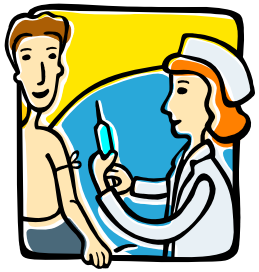
Drug: peptide

- Tween 20 is needed to counteract the non-specific binding (NSB) in saline:
  No loss of drug during five-fold transfer between polypropylene (PP) tubes when adding **0.2% Tween 20**
- Addition of Tween 20 to BAL Fluid immediately after collection is not possible (possible cell disruption)

➢ Use NALGENE conical bottom centrifuge bottles, **polypropylene copolymer**, as BAL pooling and supernatant containers
→ **reduced NSB**

➢ Addition of Tween **to ELF after centrifugation**

- Use low bind polypropylene tubes and DWPs, avoid glass.
- Use a content of at least 20% organic solvent in solutions, avoid pure aqueous.



Practical recommendations for clinical studies

- **Develop method well in advance**
  - Test plasma and whole blood **stability**
  - Perform **NSB test** for urine and other required matrices (e.g., saliva)
- Use stabilizers and agents to prevent NSB only when needed – not just in case
- If possible, **use commercial collection tubes**;
  (consider customized tubes preparation only if stability tests fail)
- Check at which stage stabilizer has to be added (e.g., blood or only plasma tube)
- If possible, **avoid toxic stabilizers** or reduce amount
  - Use **alternative stabilizers**
  - Employ “cold workflow” (challenge: ***temperature control!***)
  - Perform sample prep on site (e.g., protein crash at sampling)
    Challenge: ***whole blood method may be needed!***
- **Validate** variable amounts of stabilizer if needed (e.g., variable urine volumes)
- Investigate/validate variable blood volumes if needed (e.g., 0.5 mL in 2 mL tube) – variable anticoagulant/stabilizer, possible hemolysis
- **Provide detailed instructions & check procedures on site**, particularly when non-standard procedures need to be used

Acknowledgments

- F. Hoffmann-La Roche

  Roche Innovation Center Basel:

  Pawel Dzygiel
  Enric Bertran
  Elke Zwanziger
  Andreas Gloge
  Julian Potter
  Daniela Fraier
  Shaolian Zhou
  Lisa Benincosa
  Luca Ferrari
  Franz Bucheli
  Denis Herzog
  Thomas Wirz
  Ruedi Erdin
  Massimiliano Donzelli

  Roche Innovation Center New York:

  Faye Vazvaei
  Surendra Bansal

- European Bioanalysis Forum Topic Team 28 – Stabilization of samples in practice:

  Peter von Amsterdam
  Martijn Hilhorst
  Richard Abbot
  Silke Lüdtke
  Elke Zwanziger
  Katja Heinig

Recommendations published in:
Bioanalysis (2015) 7(3), 333