

# Stabilisation of Clinical Samples

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***on behalf of the EBF TT-28***  
***(Stabilisation of samples in practice)***

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# Introduction

## ➤ Scope

- Stabilisation of samples for quantitation of unstable drugs (and/or their metabolites) in biological fluids such as plasma, blood and urine. Small molecules, peptides and small proteins are considered.
- Prevention of nonspecific adsorption for urine.
- Feasibility / practicality of stabilization approach, i.e. in a clinical setting.

## ➤ Goal

- Collect examples for stability issues, their solution and challenges from EBF members.
- Perform literature search on stabilisation of unstable compounds
- Collect information on commercial / custom made sample collection devices.
- Provide recommendations.

## ➤ Outcome

- Extensive summary table on stabilisation of a variety of unstable compounds (feedback from 16 EBF members, literature) is being created.
- Supply of commercial / customized sample collection tubes.
- Recommendations from TT-28 on sampling procedures/devices to possibly overcome some practical challenges in stabilisation.

# Unstable compounds overview\*

Compound	Issue
Acylglucuronide metabolites	Hydrolysis to parent drug, acylmigration
Lactones (e.g. statin drugs)	Reversible hydrolysis to hydroxy acid
Prodrugs (alkyl esters)	Conversion into active drug by cleavage of ester bonds by esterases
Amides	Hydrolysis to acid
Oxidizable compounds (folates, catecholamines)	Oxidative degradation
Peptides/small proteins (neuropeptides; hormones; GLP1 analogues)	Potential dipeptidyl peptidase IV catalyzed N-terminal degradation
Cytostatic nucleosides (Cytarabine, Gemcitabine)	Enzymatic deamination
Thiols (captopril)	Dimerisation
Enantiomers	Racemisation
Cis/trans isomers	Interconversion
Alkylating agents (anticancer drugs)	Unexpected metabolic transformations

\*examples from EBF community

# Overcoming stability issues

- Acidification
- Addition of stabilisers (enzyme inhibitors, anti-oxidants, reducing agents)
- Rapid processing of blood; cooling; cold storage (e.g. -70°C) ; light protection
- Immediate sample preparation (blood crush; derivatisations, i.e. methyl acrylate)
- Dried Blood Spots (not further discussed here)

# Challenges (clinical environment)

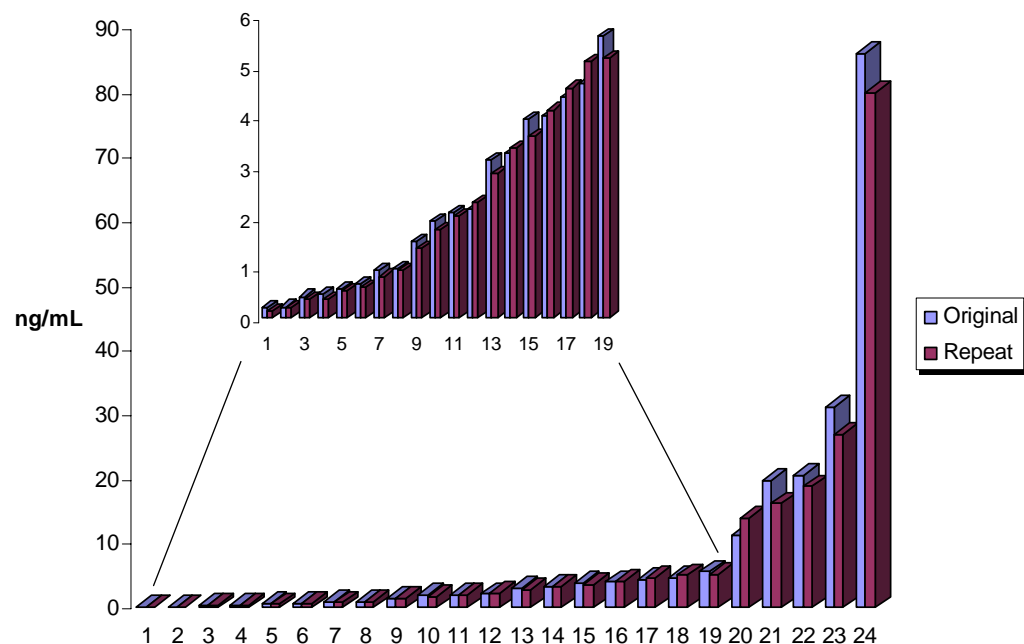
- Blood cannot be acidified to pH <5 : coagulation, plasma gelling
- Proper pH crucial for stability (i.e. with interconverting compounds)
- Time critical processes have to be managed (rapid plasma preparation, rapid transfer to freezer)
- Availability of cooled centrifuges and ultrafreezers (-70°C or below)
- Commercial availability & costs of tubes containing stabilisers
- Safety: handling of highly toxic esterase inhibitors (dichlorvos) and strong acids (HCl, formic acid) in the clinic
- Some additives not stable (tetrahydrouridine, aprotinine); need to be added into sampling device at day of sampling
- Exact pipetting of stabilisers to sample tubes may be difficult for clinical staff

# Real case example 1:

## Compound X (ester prodrug)

- ISR in 7 day non-GLP MTD study failed (100% of prodrug ISR samples failed)
  - Rat blood collected into K2EDTA and chilled on ice
  - Plasma decanted into tubes containing inhibitor cocktail including 5% 20mM citrate buffer (pH6)
- **Investigation :**
  - Assay Validation had worked fine; it was noted that in order to solubilise the reference material, spiking standards were prepared in 0.5M citric acid
  - Subsequent testing demonstrated that if the inhibitor cocktail was changed to 5% 0.5M citric acid, the prodrug was successfully stabilised

- ISR in 28 day rat GLP study with revised 5% 0.5M citric acid inhibitor :  
Successful ISR data demonstrated below



# Real case example 2:

## Compound Y (acyl glucuronide)

- Unstable in human blood with EDTA or citrate (commercial tubes) for 15 min at 37°C.
- **Stable** in acidified blood (EDTA, citric acid added to pH 5.6) for at least 1 h at RT.
  - pH <5.0: blood coagulates,
  - pH <5.5: strong haemolysis
  - pH >6.2: compound unstable
- Unstable in human plasma (from citrate blood) for 1 hour at 0°C or RT.
- **Stable** in plasma from acidified blood (pH range 4.5 to 6.2 checked) for 16 h at RT (and 3 months at -20°C, 5 x freeze/thaw).
- Only stable in sample solution if it contains 0.2% formic acid.

➤ Challenge in clinic:



(Courtesy A. Gloge, Roche)

To avoid handling of stabiliser in clinic, vacutainers were pre-treated with citric acid and send to facility. Citric acid solution was highly concentrated to keep sample dilution negligible (0.75% volume-spike).

Blood acidification by preloading sampling tubes failed: Citric acid crystallized in vacutainers, blood coagulated on the surface of crystals and prevented them to be further dissolved.

Solution: Add citric acid solution manually to each tube, directly after blood sampling.

# TT Recommendation – Blood/Plasma (1)

- If possible, use commercially available sampling tubes:
  - NaF/K-oxalate
  - NaF/EDTA
  - NaF/heparin
  - Citrate
  - NaF/Citrate + NaF
  - RNA protect
  - DNA protect
  - P700 (DPPIV inhibitor)
  - Protease blockers (e.g. phenylmethylsulfonyl fluoride (PMSF) + eserine (physostigmine))
  - Acid citrate dextrose
  - EDTA (can have a stabilizing effect next to being an anticoagulant)
  
- Vendor preparation of customized pre-treated tubes with stabilisers to avoid addition in the clinic
  - Minimum order 50'000 tubes for € 10'000 (not practical; storage & stability?)
  - Proposal: several companies together (EBF community) may order same tubes
  - Note: some stabilisers cannot be added by machines (i.e. surfactants create foam)
  - Import of tubes to US difficult (medical device, approved by FDA)
  - **Haematologic Technol. Inc. prepares custom tubes in US (non sterile), 3-5 USD/tube**

# TT Recommendation – Blood/Plasma (2)

- In-house/on-site preparation of tubes with stabilisers
  - A) Pre-sampling added to tubes with syringe. Vacuum tubes without any additive can be purchased. Use small needle to prevent losing vacuum. 10 µL/ mL blood.
  - B) Post sampling added to blood tube and/or plasma tube.

## Available stabilisers (selection):

Stabilizer class	Examples	Compound class
Esterase/protease inhibitors	NaF, phenylmethane-sulfonylfluoride [PMSF], bis(4-nitrophenyl)-phosphate [BNPP], eserine, paraoxon, acetylcholine, dichlorvos, Ellman's reagent, diisopropylfluorophosphate [DFP]	<i>Ester prodrugs, amides</i>
Acids	pH~3 formic acid, HCl; pH~4 o-phosphoric acid; pH~5 citric acid	<i>Acylglucuronides, lactones, esters, amides, enantiomers</i>
Anti-oxidants	ascorbic acid, mercaptoethanol/propanol, Na-metabisulfate, L-cystein; EDTA as anticoagulant	<i>Folates, catecholamines</i>
Reducing agents	dithiothreitol, pyrosulfit	<i>Thiols</i>
Alkylating agents	Methyl acrylate, N-ethyl maleimide	<i>Thiols</i>
Enzyme inhibitors	Aprotinine (DPPIV inhibitor)	<i>Peptides/proteins</i>
	Dihydrouridine, THU (cytidine deaminase inh.)	<i>Cyteostatic nucleosides</i>
Buffers	Tris pH 9	<i>Indolequinones</i>



# TT Recommendation – Blood/Plasma (3)

## ➤ Further recommendations

- If possible, avoid use of toxic stabiliser compounds or reduce amount, check alternatives (e.g. F-oxalate instead of dichlorvos) or use low temperature (cool blood tubes before blood withdrawal & put on ice)
- Check if stabiliser has to be added to blood or only to the plasma tube
- Consider whole blood method with protein crash immediately after sampling
- Provide detailed instructions to the clinic, train nurses, check procedures on site

## ➤ Regulatory concerns

- Bioanalytical method validation
  - o Plasma used during validation should contain same amount of stabiliser as the collection tubes used in routine bioanalysis; several tubes are available containing different amounts of stabilizer.
  - o If constant amount of stabiliser is needed: accurate pipetting is needed; go to clinic to check if they do it correctly
  - o For sample processing, stick to validated procedure i.e. blood 30 min on ice
  - o Add/change stabiliser: consider extent of required method validation
- GCP issue: expiry date on tubes required; not available for custom made tubes and needs to be tested before use

# URINE – a Different Story

## ➤ Issues

- Chemical instability of analyte/metabolite
- Apparent instability caused by nonspecific binding (adsorption in collection/ storage/ sample preparation vessels), particularly with hydrophobic small molecules, bases & peptides/proteins
- Apparent instability/irreproducibility due to adsorption onto precipitates

## ➤ Solution

- Control pH by adding acid/base/buffer
- Use additives to prevent nonspecific binding
  - o BSA (good for compounds with high protein binding)
  - o Surfactants, e.g. CHAPS, SDBS, 0.1% (v/v) Triton-X 100 or Tween 80
- Use low bind tubes, plastic (PP, PE) vs. glassware

## ➤ Challenges

- When should the additive be introduced to the urine?
  - o Pre-collection: safety/regulatory concerns because subjects would handle the additive; variable urine volumes create variable detergent concentrations
  - o Post-collection and/or post-transfer: compound loss possible in particular with multiple transfers; not all PP or PE show the same binding
- Additive may impact determination of clinical parameters
- In case of reproducibility issues, each sample has to be aliquoted manually immediately after homogenization (vortex or ultrasonic; centrifugation can lead to analyte loss) which limits the use of robots. Analytical lab in charge.

# TT Recommendation – Urine

Three situations can occur when adding stabilisers:

- The compound of interest sticks to the container but can easily be desorbed:  
Add an amount (for example 1%) of the required solution (containing Tween, BSA or other agent) after the collection of all periods of a certain time interval (for example 0-24 hours). This requires weighing.
- The compound cannot be easily desorbed or is unstable:  
To each portion of produced urine, directly add an amount of solvent/stabiliser (labor intensive).
- The compound is very unstable or sticks irreversibly to the container  
Additive needs to be added before urine is collected, volunteer urinates on top of the solution. Required percentage of additive is known but not the amount of urine that will be produced.

Suggestion for validation: Investigate min/max concentration of additive, all percentages in between are then considered covered.

Review on available additives and proposal for experiments:

Ji, Jiang, Livson, Davis, Chu, Weng; **Challenges in urine bioanalytical assays: overcoming nonspecific binding**; Bioanalysis (2010) 2 (9), 1573

# Literature

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