Assessment of ISS using an efficient standardized stepwise “black box” process.

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Description of the problem

- High focus on Incurred Sample Reproducibility (ISR) in industry
- Incurred Sample Stability (ISS) is just as important
  - Is this addressed sufficiently ?
  - Can this be addressed sufficiently ?

Stability assessments in spiked matrix do not always reflect stability assessments in incurred samples (ISS) - Why ? - 1

- Presence of metabolites in incurred samples is main reason
  - Some metabolites degrade to unchanged drug (UD)
    - During sampling (in blood, plasma, urine..)
    - During sample storage
    - During sample processing
    - In the auto sampler
    - In the ion-source
    - On the HPLC-column
  - Some metabolites are isobaric with UD
  - Some metabolites cause ion suppression of UD or internal standard due to co-elution and thus impact quantification of UD
- **All of the above could also potentially lead to ISR failures !**

Stability assessments in spiked matrix do not always reflect stability assessments in incurred samples (ISS) - Why ? - 2

- … And more ?
  - Plasma used for spiked matrix stability not always fresh, thus less enzymatic activity
  - Differences in recovery due to differences in protein binding (probably rare as often the binding is disrupted in the sample prep ?)
  - Differences in matrix effect (compensated for if stable label internal standard is used)
  - Etc

The regulatory landscape

- FDA Guidance 2001
- Xtal city 3
- EMA 2011 (seems the most extensive with regard to ISS)

All the above documents touch ISS, but not fully integrated as ISS (part of other paragraphs)

The regulatory landscape – 1

FDA Guidance 2001

5. Post-Preparative Stability

- Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of an analyte's stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

E. Principles of Bioanalytical Method Validation and Establishment

- For compounds with potentially labile metabolites, the stability of analyte in matrix from dosed subjects (or species) should be confirmed.

The regulatory landscape – 2

Xtal City 3 workshop report AAPS Journal 2007, 9 E30-E42

- INCURRED SAMPLE RE-ANALYSIS
  - "There are several situations where the performance of standards and QCs may not adequately mimic that of study samples from dosed subjects (incurred samples). Examples include metabolites converting to the parent species, …"
  - "… These could include metabolites converted to parent during sample preparation or LC-MS/MS analysis, matrix effects from high concentrations of metabolites, …"

The regulatory landscape – 3

EMA Guideline on bioanalytical method validation - 2011

- **4.1.1. Selectivity**
  - "It may also be necessary to investigate the extent of any interference caused by metabolites of the drug(s) "
  - "The possibility of back-conversion of a metabolite into parent analyte during the successive steps of the analysis (including extraction procedures or in the MS source) should also be evaluated, when relevant (i.e. potentially unstable metabolites e.g. acidic metabolites to ester, unstable N-oxides or glucuronide metabolites, lactone-ring structures). The extent of back-conversion should be established and the impact on the study results discussed. It is acknowledged that this evaluation will not be possible early during drug development of a new chemical entity when the metabolism is not yet evaluated. However, it is expected that this issue is taken into account and a partial validation is performed if relevant as further knowledge regarding metabolism of the active substance is gained during drug development. "
  - "It is recognized that in some cases it is very difficult to obtain the metabolites of interest. Alternatively, back-conversion of a metabolite can be checked by applying incurred sample reanalysis. However, in this case potential back conversion during sample processing cannot be ruled out. "
- **4.1.9. Stability**
  - *"Regarding long term stability of the analyte in matrix stored in the freezer: …* Study samples may be used in addition to QC samples, but the exclusive use of study samples is not considered sufficient as the nominal concentrations of those samples is not known. …"

Challenges in the assessment of influence of metabolites on assay performance

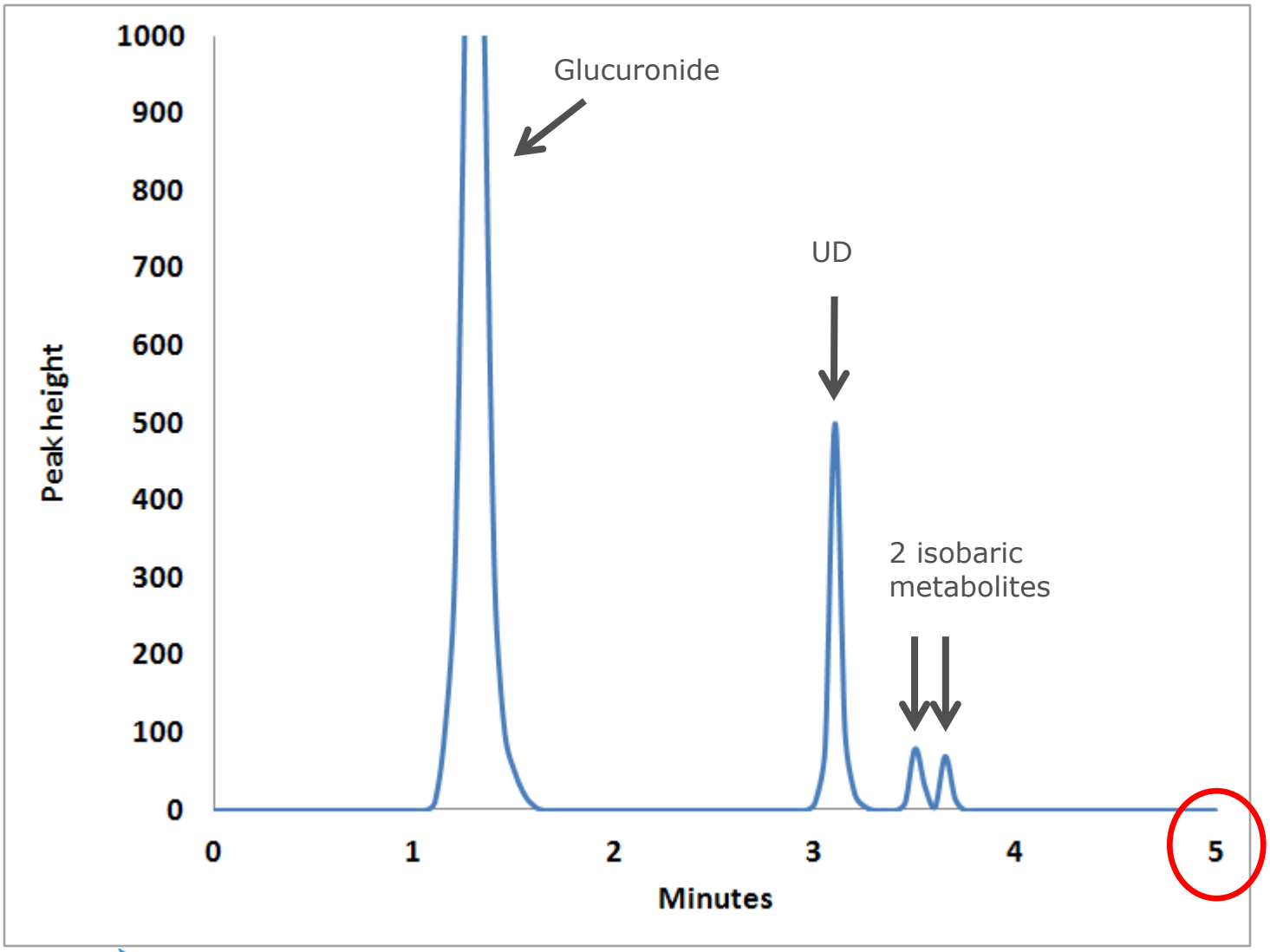
- Metabolite profile (both qualitative and quantitative) often not completely known
- Relative abundance of metabolites is important:
  - Metabolite A = 10x UD
    → 5% back conversion to UD → 50% overestimation of UD levels.
  - Metabolite B = 0.1x UD
    → 50% back conversion to UD → 5% overestimation of UD levels.

Although info on metabolite profile and abundance is very useful, it is not required when incurred samples are available

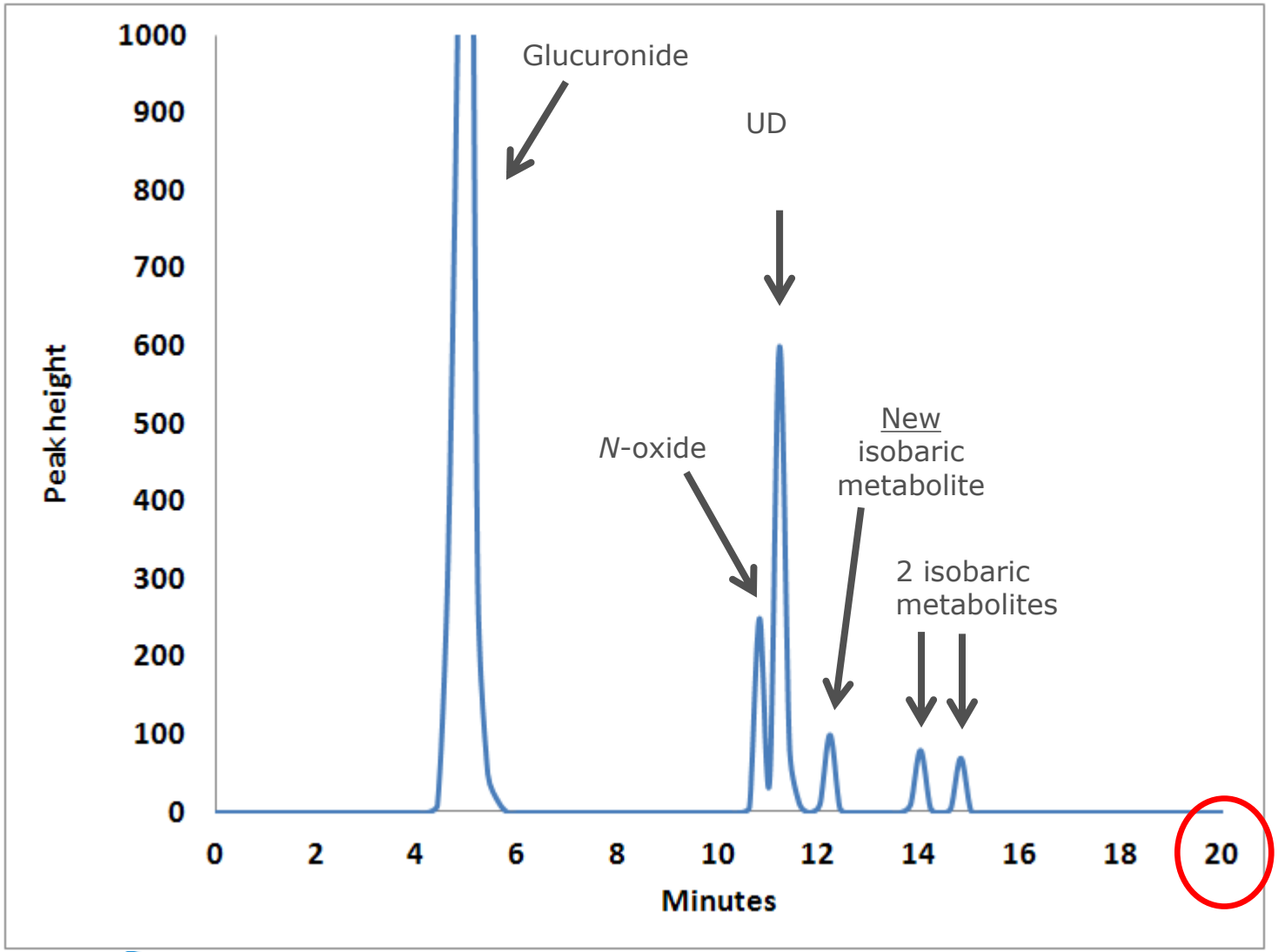
→ the 'black-box' process

It’s time for an example ….

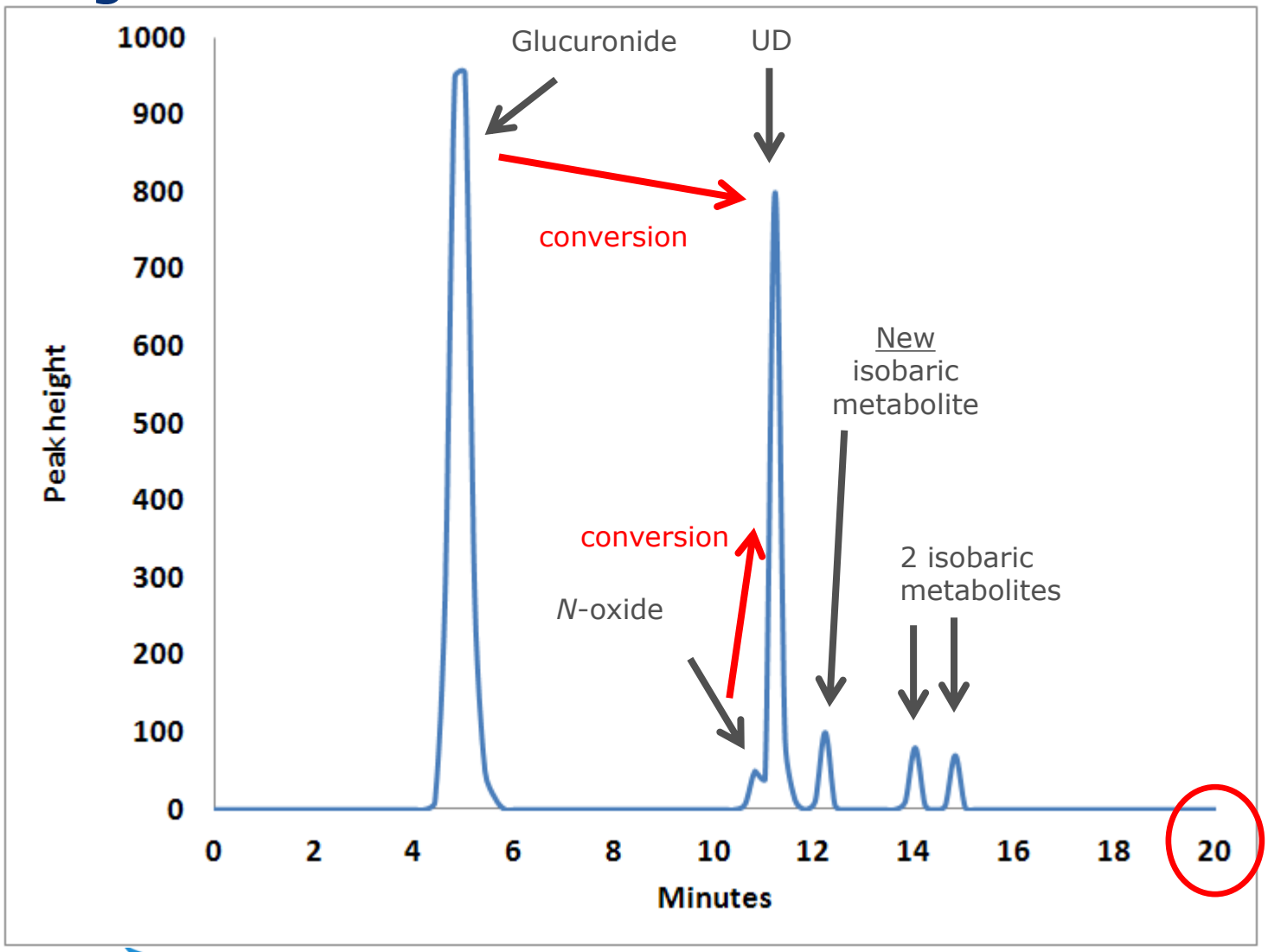
5 minute run


Glucuronide
UD
2 isobaric metabolites
Peak height
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
Minutes

Slow chromatography

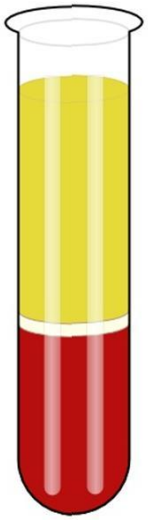


Degradation issues



The ‘black-box’ process

Step 0 Sample selection



Step 1

Co-elution

Step 2

Sample prep

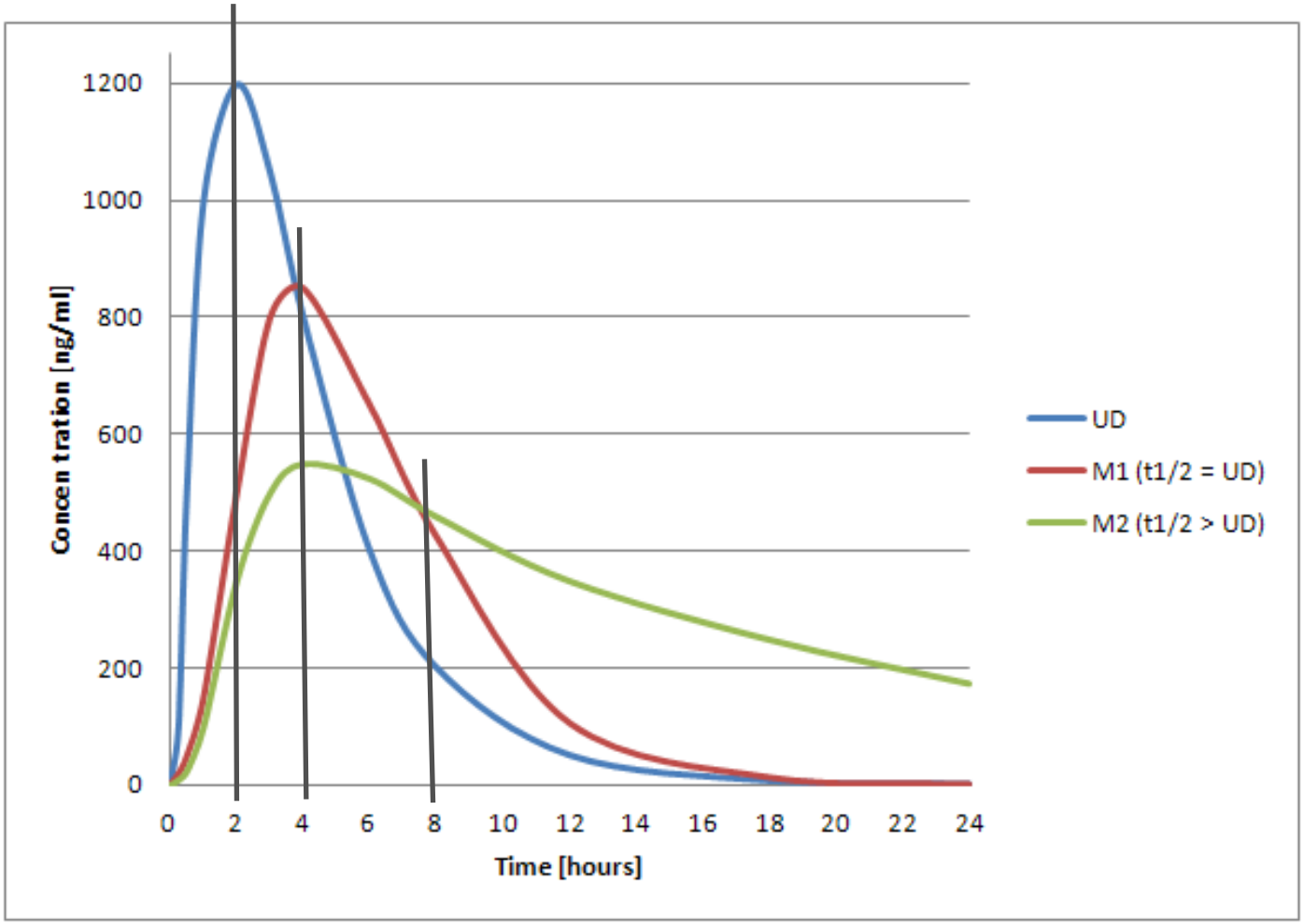
Step 3

Stability in matrix

Step 4

Ion suppression

Step 0: sample selection

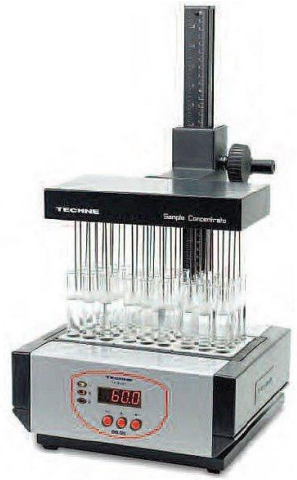


Step 1: Check for co-elution

- Slow chromatography
  - (preferably UPLC), e.g. 20-30 minute gradient.
  - consider doing this at 2 # pH's
- Evaluate chromatograms of whole analytical run
  - do additional peaks appear after multiple incurred sample injections ?
- Actively search for expected 'problem' metabolites
  - based *on in vitro / in vivo / in silico* metabolism data
  - add the MRM transitions, e.g.:
    - +16 (*N*-oxide)
    - +80 (sulphate)
    - +176 (glucuronide)

Step 2: check the sample preparation

- Stress test the sample preparation
  - In time
  - High pH, low pH
  - Heat
  - Protein precipitation (no heat, high or low pH) as comparison
  - Include a spiked sample for comparison



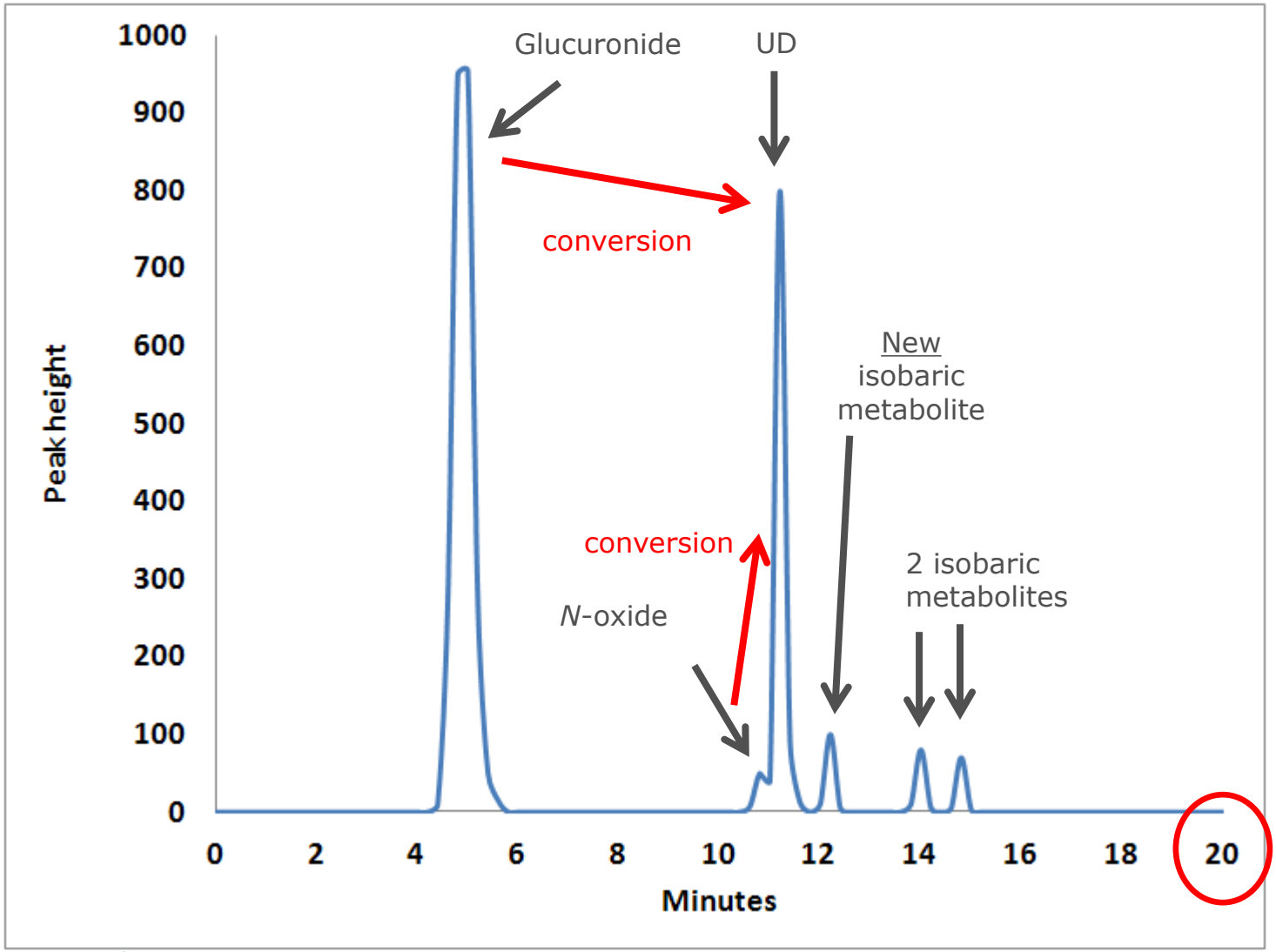
Step 3: Check stability in matrix (plasma, blood)

- This step can only be done when Step 1 and 2 are completed successfully
- Perform on-the-bench stress test storage
  - e.g. 4 hours and overnight
  - Include a spiked sample for comparison

Step 4: Assess ion suppression

- Focus on suppression of UD or IS caused by co-eluting metabolites:
  - this step can be skipped in case of stable label IS
- Compare slow chromatography with validated assay (faster chromatography):
  - if same result then probably no issue
- Monitor IS response:
  - high variation is indication for suppression issues
- Include UV-detector (LC-UV-MS/MS), and/or run precursor ion and Q1 scan to check for co-eluting metabolite peaks

Back to the example…



Timing of the experiments

- Pre-clinical:
  - Preferably as part of method development (= prior to method validation), if incurred samples from pre-GLP studies are available
  - If no incurred samples are available at pre-validation stage, perform 'black box' after first animal study
  - Experiments are needed per species
- Clinical:
  - As soon as possible after samples from the first into human have been analyzed

Reporting of the data

- Reporting of the data less straightforward
  - Some data not obtained with the validated assay (protein precipitation as alternative sample preparation; slow chromatography, etc)
- Results obtained with incurred samples important as supporting evidence that method is producing valid results → method validation report seems the logical place for reporting, although currently not common practice
  - Current practice @ Janssen: document in raw data of method development – no report
  - Might evolve into reporting in method validation report
- Discussion in industry is useful !

Conclusion

- ISS is needed above ISR
- Conduct of ISS is difficult
  - Innovative thinking allows a solution
- More discussion needed in industry

Acknowledgement

- Philip Timmerman (for the discussions, review and input)

- Many colleagues that helped to build the 'black box' process over the past years through their projects and method development work
  - Tom Verhaeghe, Marc De Meulder, Hans Stieltjes, Vera Hillewaert, Petra Vinck, Iris Vanwelkenhuysen, Lieve Dillen, Luc Sips, Luc Embrechts, Willy Lorreyne, Ief Vandermot, Tinne Huybrechts, Marc Verhemeldonck, Luc Diels, Ils Pijpers