



»» TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY

Importance of End-to-End Robustness when dealing with Glucuronide Metabolites

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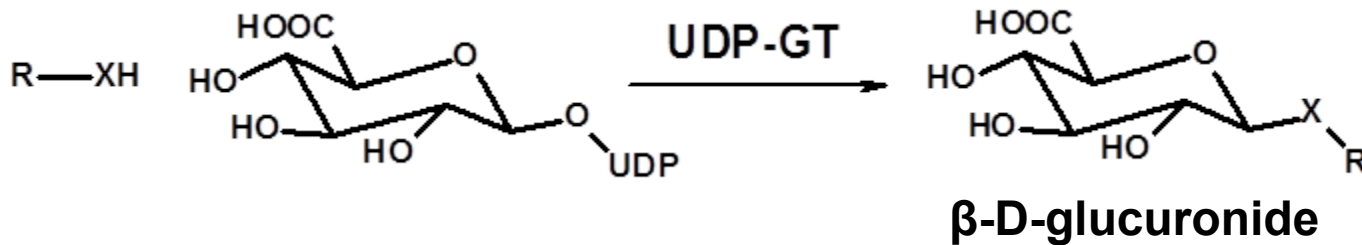
Agenda

- Introduction
- Biological samples
- Samples analysis
- Samples extraction
- Quantification of glucuronides
 - > Indirect
 - > Direct
- Conclusions



Introduction

- Glucuronidation is a major metabolic detoxification pathway
 - > Formation of inactive water-soluble molecules \Rightarrow **Excretion**
 - > Metabolite more active than the parent drug \Rightarrow **Bioactivation**
- Conjugation process = Nucleophilic xenobiotic molecule + glucuronic acid



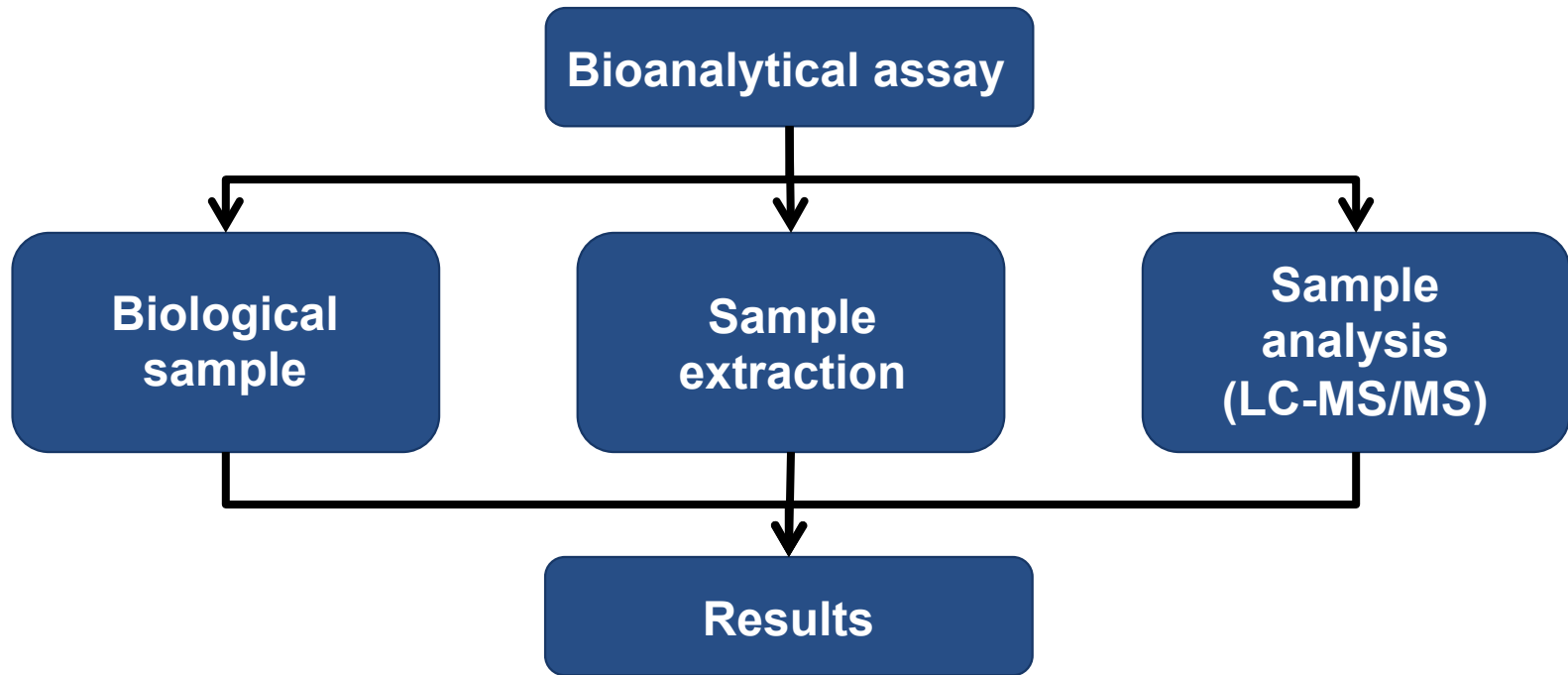
| Functional Group (XH) | | Product |
|-----------------------|-------------------------------------|------------------------|
| Carboxylic acid | R-COOH | Ester |
| Alcohol | R-OH | Ether |
| Thiol | R-SH | Thioether |
| Amine | RNH ₂ , R ₃ N | Aminoketal or 4° amine |

-
↓
+
Hydrolysis
Stability



Introduction

- Bioanalytical assay robustness
 - Ability to behave under multiple conditions to generate reliable results.

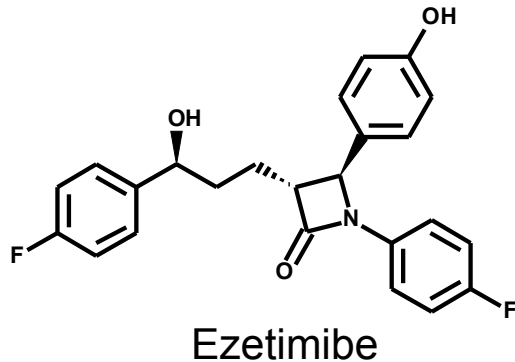


- Back-conversion of metabolites at every step of the assay must be evaluated
 - Improve reliability of the assay to deliver accurate concentration of unconjugated analyte in incurred samples.



Biological sample

- Evaluate the impact of the glucuronide metabolite on the measurement of unconjugated drug
 - > Various stability tests in matrix in presence of metabolite.



80-90% of total drug as O-glucuronide (phenol group)

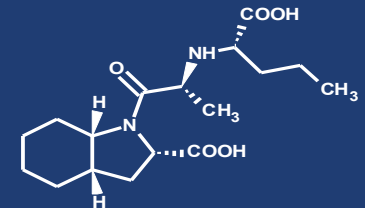
- Low and high QCs fortified with metabolite
 - > Estimated C_{max} concentration.
 - > Estimated physiological concentration.

Tests

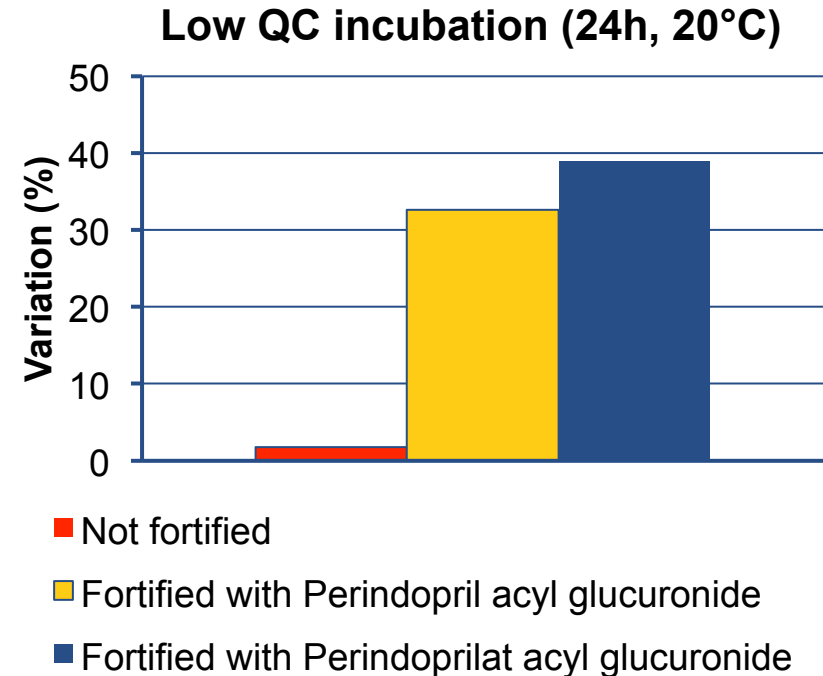
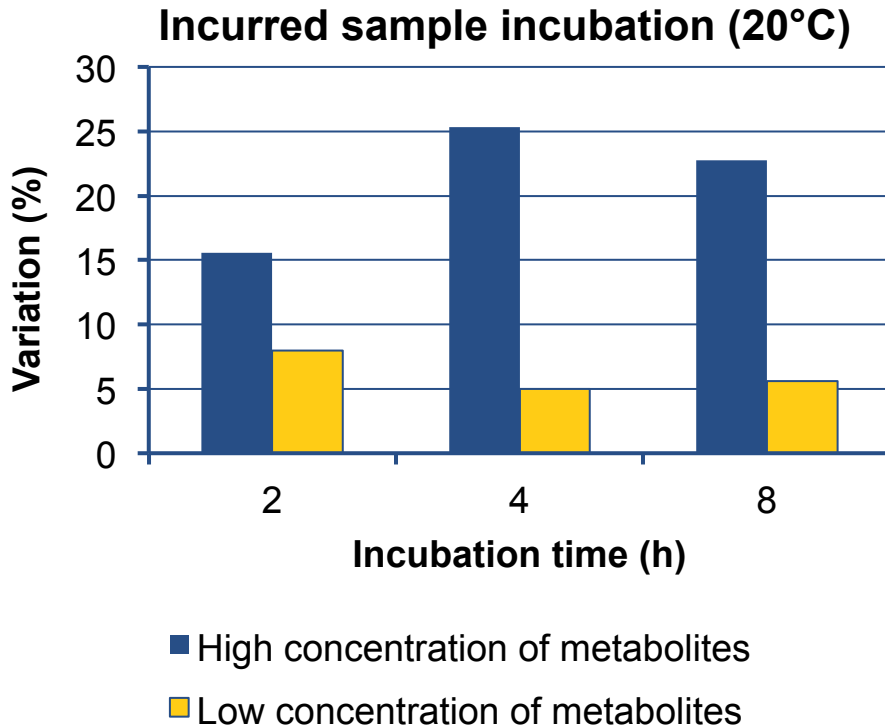
| |
|--------------------------------|
| Short-term stability in matrix |
| Long-term stability in matrix |
| Freeze and thaw stability |
| Post-preparative stability |
| Whole blood stability |
| Incurred sample reanalysis |



Biological sample: case study



- Perindoprilat: incurred sample reanalysis inaccuracy



- Perindoprilat results were inaccurate for sampling times 0.3h to 3h
 - > Back-conversion of metabolites into Perindoprilat in plasma samples.
 - > Method was modified to avoid the back-conversion.

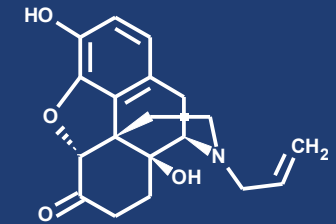


Sample analysis (LC-MS/MS)

- Evaluate the potential fragmentation of the metabolite at the detection step to avoid over-estimation of the parent concentration
 - > MS ion source (ESI or APCI).
 - > Collision cell (ion channel cross-talk).
- Develop good chromatographic separation conditions of parent and metabolites
 - > Avoids a false estimation caused by the fragmentation of the co-eluting glucuronide metabolite.
 - > Assess the chemical purity of the β -D-glucuronide reference standard in term of parent analyte.

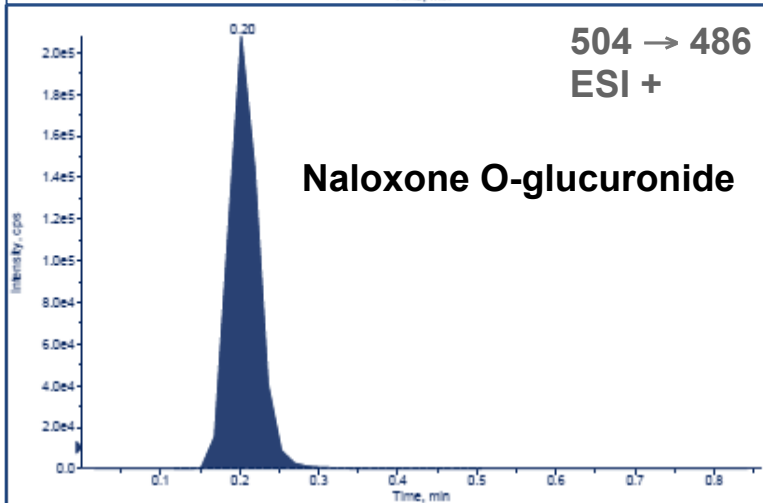
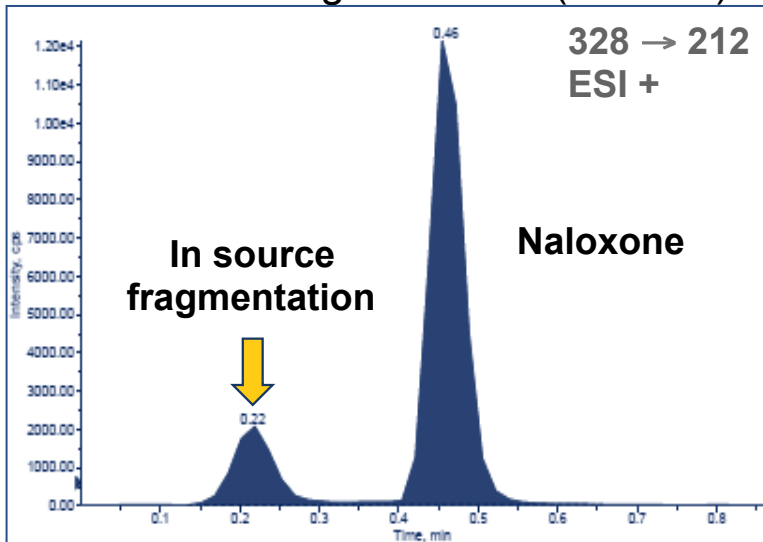


Sample analysis (LC-MS/MS)



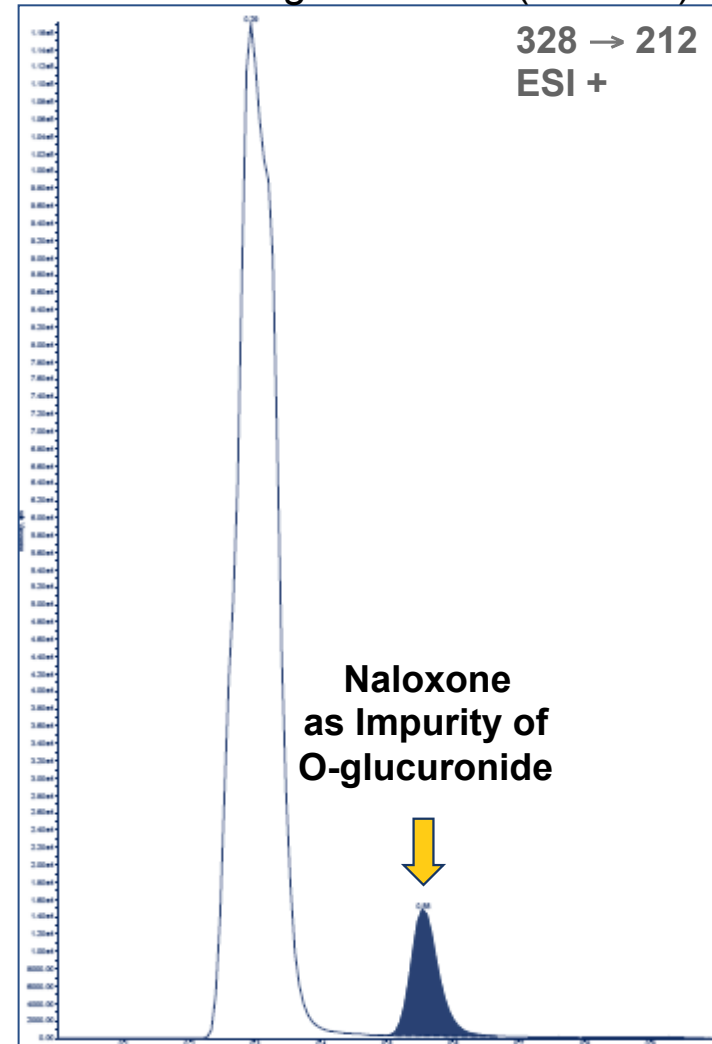
Evaluation of fragmentation

Naloxone + O-glucuronide (solution)



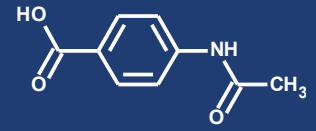
Assessment of purity

Naloxone O-glucuronide (solution)





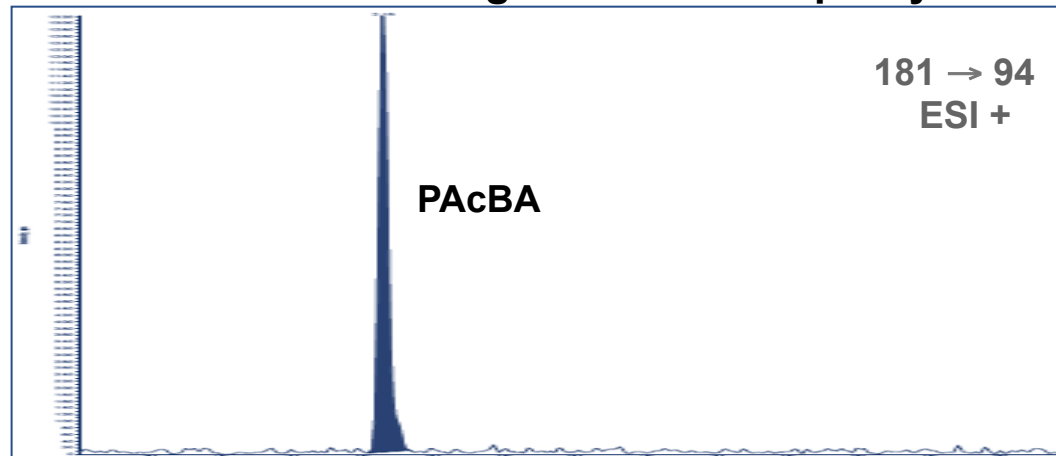
Sample extraction



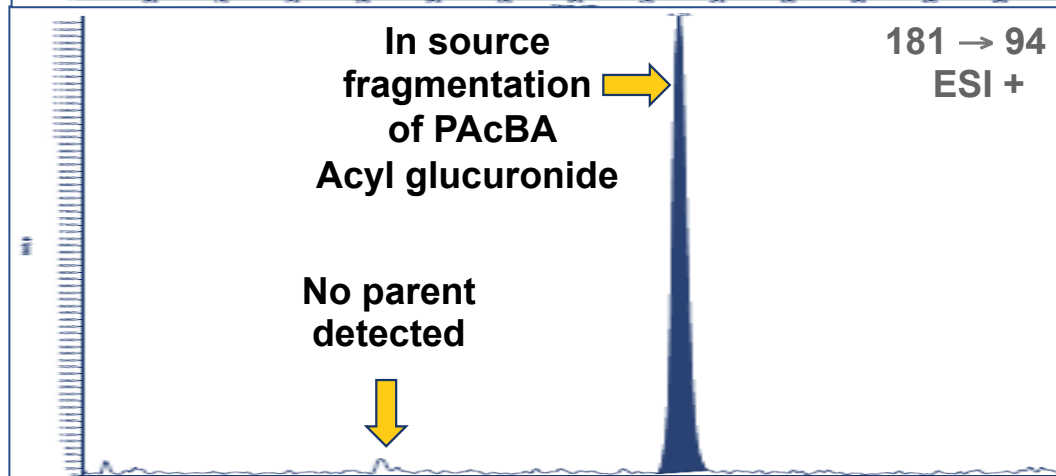
- Evaluate the potential hydrolysis of β -D-glucuronide during samples preparation in the measurement of the unconjugated drug

Evaluation of fragmentation and purity

PAcBA
(human plasma)

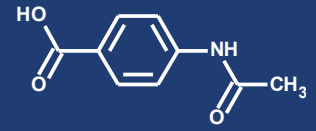


PAcBA
acyl glucuronide
(solution)

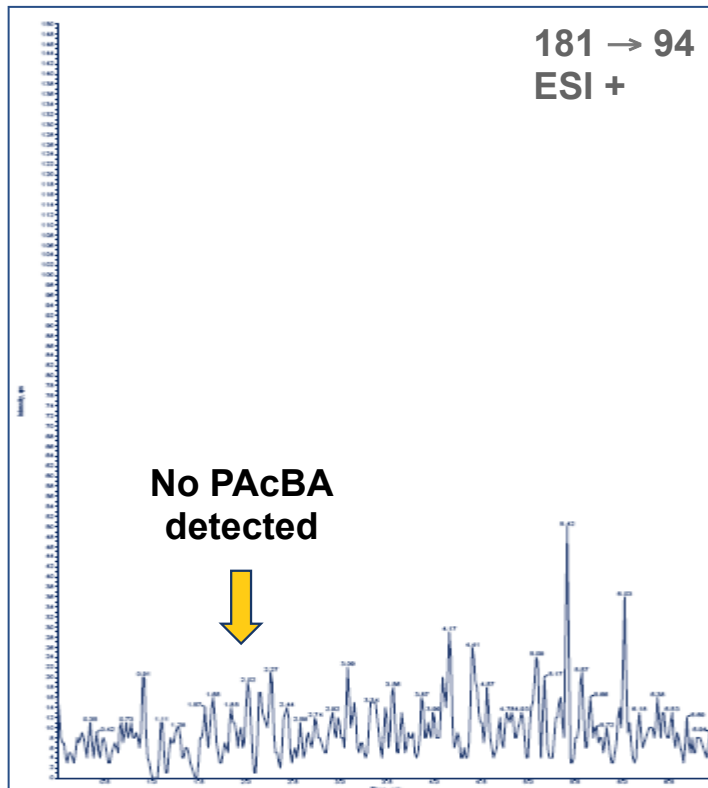




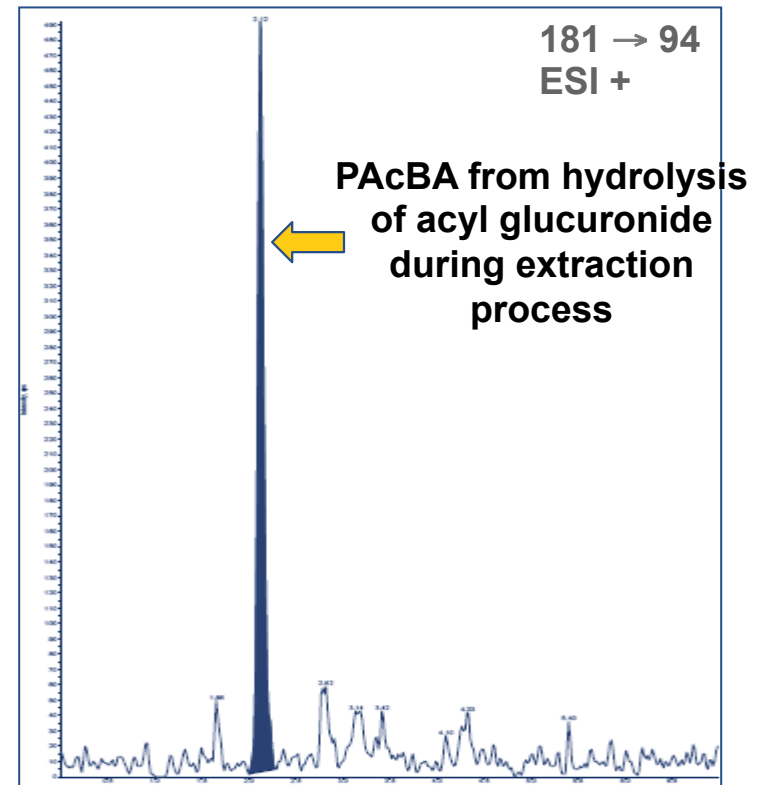
Sample extraction



Blank human plasma



PAcBA acyl glucuronide
(blank human plasma)



- Extraction conditions were modified to reduce the hydrolysis of the PAcBA glucuronide
 - Ensures accurate quantification of the parent analyte in incurred samples.



Quantification of glucuronide metabolites

- Determination of the concentration of glucuronides might be required
 - > Indirect quantification
 - Hydrolysis of β -D-glucuronide prior to sample extraction to determine total concentration of drug (unconjugated + conjugated).
 - The glucuronide concentration is obtained by calculations (total – unconjugated).
 - Hydrolysis step could be fitted in the assay of the unconjugated analyte.
 - > Direct quantification
 - Extraction and analysis of intact glucuronide.
 - Glucuronide metabolite must be stable.
 - Total concentration of drug is obtained by summation of the unconjugated drug and glucuronide.
 - New assay needs to be developed.



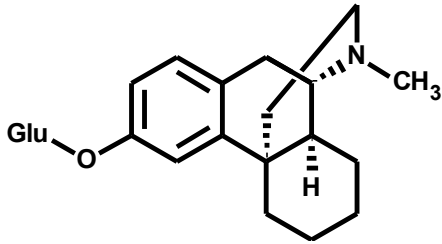
Indirect quantification of glucuronide

- Hydrolysis of glucuronides for indirect determination
 - > Enzymatic (β -glucuronidase).
 - > Chemical (acid or base).
- Glucuronide hydrolysis may be incomplete
 - > Binding to matrix components.
 - > Reactivity varies between substrates and structural isomers.
 - Concentration of enzyme or reagents.
 - Incubation temperature.
 - Reaction time.
 - > Parent analyte may be unstable in hydrolytic conditions.
- Efficient hydrolysis conditions required to ensure accurate measurement of total concentration of drug
 - > Monitoring of the hydrolysis yield is required.



Indirect quantification of glucuronide

- Conditions of the hydrolysis



Dextrorphan
O-glucuronide

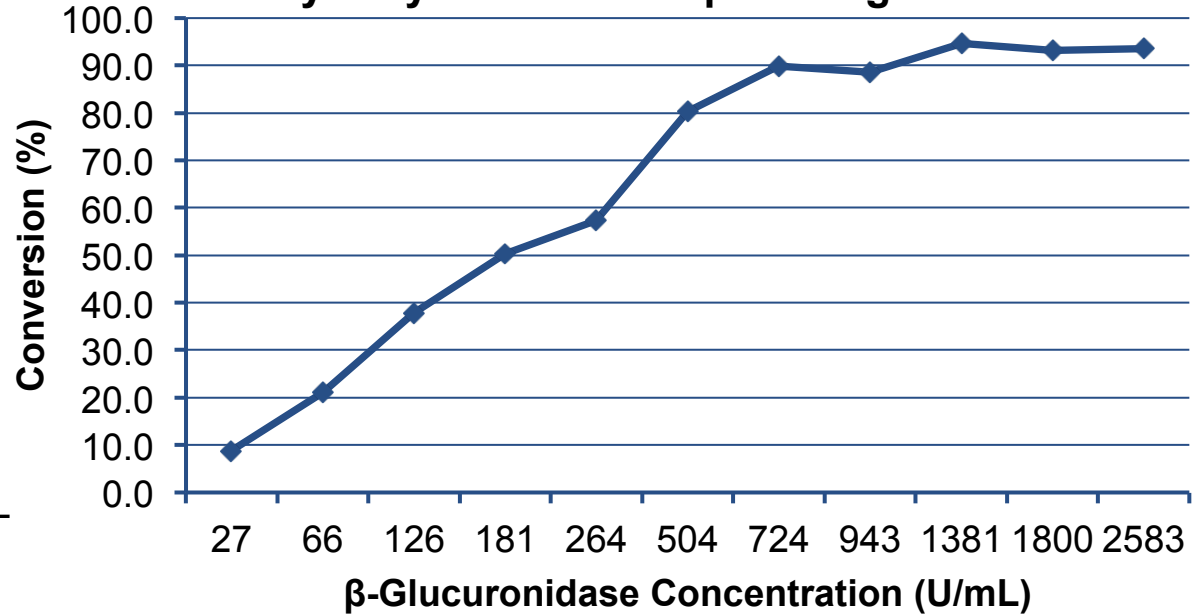
β -Glucuronidase from *E.coli*

Human plasma

Glucuronide conc. = 20 ng/mL

Incubation: 1h, 50°C, pH = 7

Hydrolysis of Dextrorphan O-glucuronide

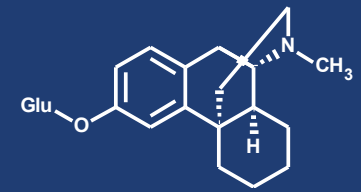


- Monitoring of the efficiency of hydrolysis within an analytical batch

| | Calibration | Quality control |
|------------|--------------|-----------------|
| Approach 1 | Unconjugated | Glucuronide |
| Approach 2 | Glucuronide | Glucuronide |



Direct quantification of glucuronide



- Direct analysis of intact Dextropropoxyphene O-glucuronide by protein precipitation
- Dextropropoxyphene O-glucuronide is stable
 - > During and after the extraction process.
 - > In plasma at room temperature (22h).
 - > After freeze and thaw cycles (4 cycles).
 - > In whole blood at 4°C (4h).

| Accuracy and Precision | QC | Conc. (ng/mL) | Accuracy Bias (%) | Precision CV (%) |
|-------------------------------|--------|---------------|-------------------|------------------|
| Inter-assay (4 runs, N=24) | LLOQ | 8 | -3.61 | 4.94 |
| | Low | 24 | -1.32 | 4.77 |
| | Middle | 4000 | 1.15 | 3.95 |
| | High | 6000 | -0.51 | 3.57 |

- Incurred sample reanalysis (ISR) performance near 100%



Determination of total Dextrorphan in human plasma

- 60 mg b.i.d Dextromethorphan  Total Dextrorphan

| | C _{max} (ng/mL) | | |
|--------------------------|-----------------------------|-------------|-------------------|
| | Dextromethorphan | Dextrorphan | Total Dextrorphan |
| Published NDA | 3.7 | 15.9 | 733.4 |
| inVentiv Health Clinical | 5.6 | 11.4 | 1856 |

- Published NDA results
 - > Quantification of hydrolyzed Dextrorphan (no detailed protocol).
- inVentiv Health Clinical
 - > Quantification of intact Dextrorphan glucuronide.
- Was the hydrolysis efficiency properly monitored for the results published in the NDA?



Conclusions

- Glucuronide metabolites can hydrolyze back to parent drug
- The fate of the glucuronide metabolite must be evaluated at each step of the assay
 - > Sample
 - Stability in matrix.
 - > Extraction
 - Stability during and after extraction process.
 - > Analysis
 - Glucuronide fragmentation.
 - Separation of metabolite and parent by chromatography.
- Hydrolysis conditions must be monitored for indirect quantification of glucuronide
- Glucuronide must be stable enough to be extracted and analyzed directly



Acknowledgements

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