In vitro biotransformation of new psychoactive substances

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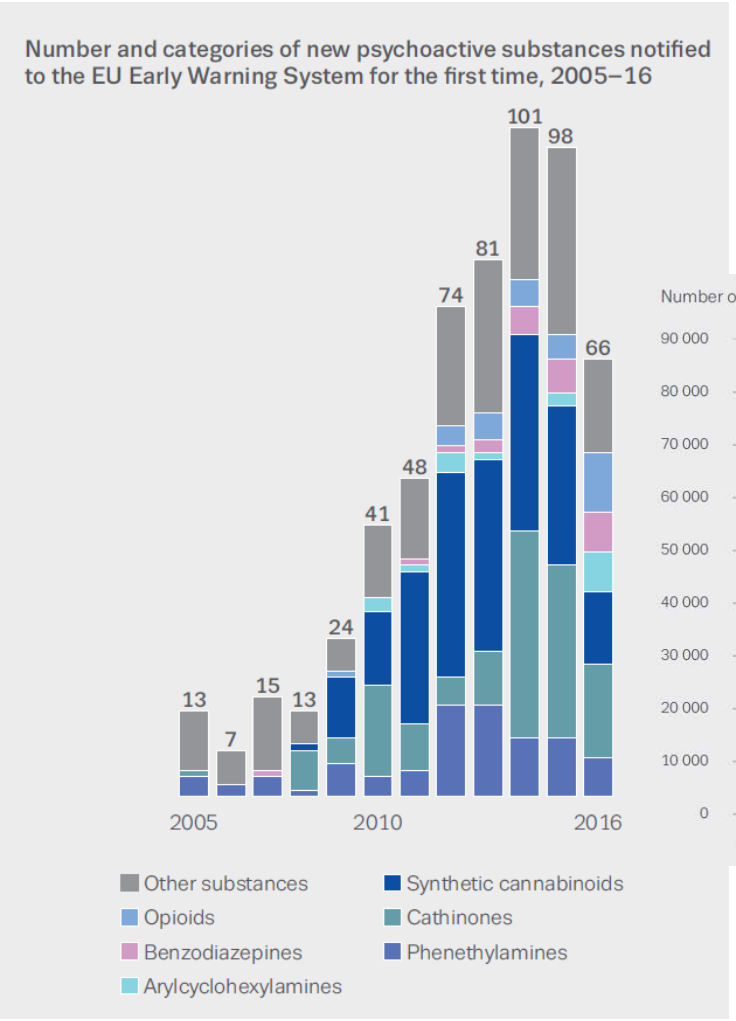
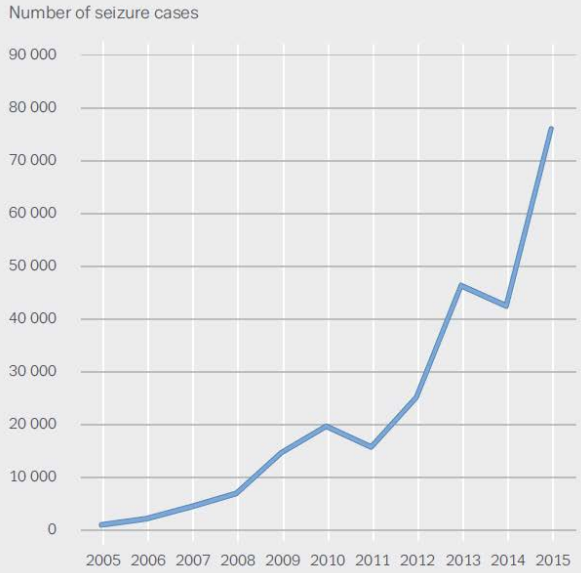
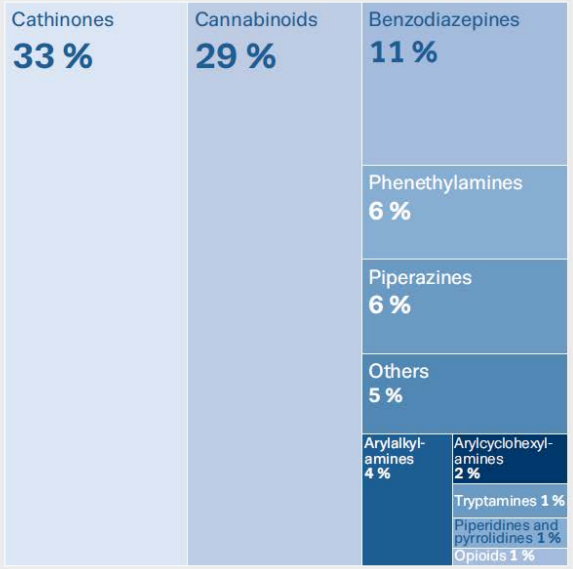
University of Antwerp

NPS in Europe…



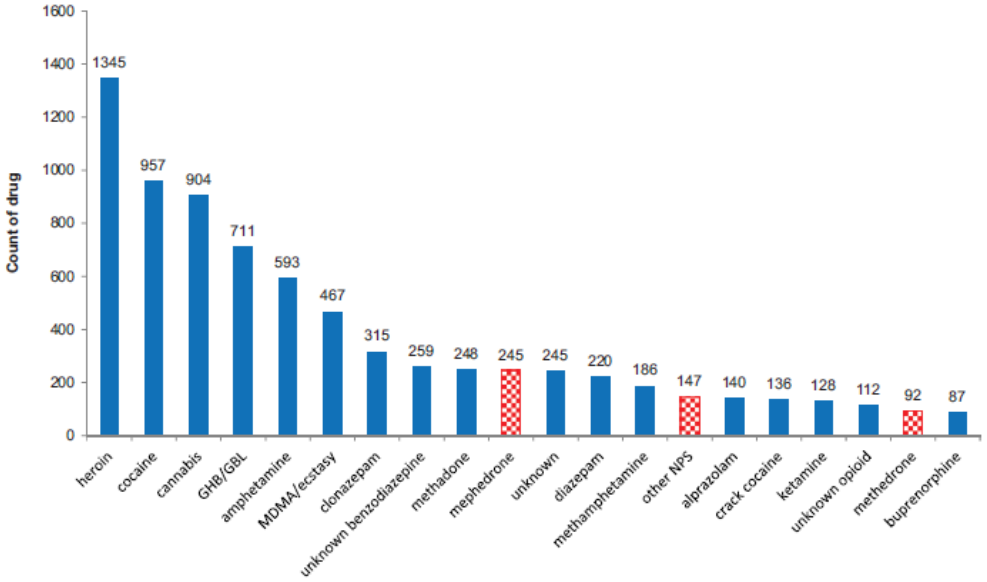




European Drug Report, 2017, EMCDDA

Acute recreational drug and new psychoactive substance toxicity in Europe: 12 months data collection from the European Drug Emergencies Network (Euro-DEN)

ALISON M DINES,[1] DAVID M WOOD,[1,2] CHRISTOPHER YATES,[3] FRIDTJOF HEYERDAHL,[4] KNUT ERIK HOVDA,[4] ISABELLE GIRAUDON,[5] ROUMEN SEDEFOV,[5] and PAUL I DARGAN[1,2]; EURO-DEN RESEARCH GROUP*



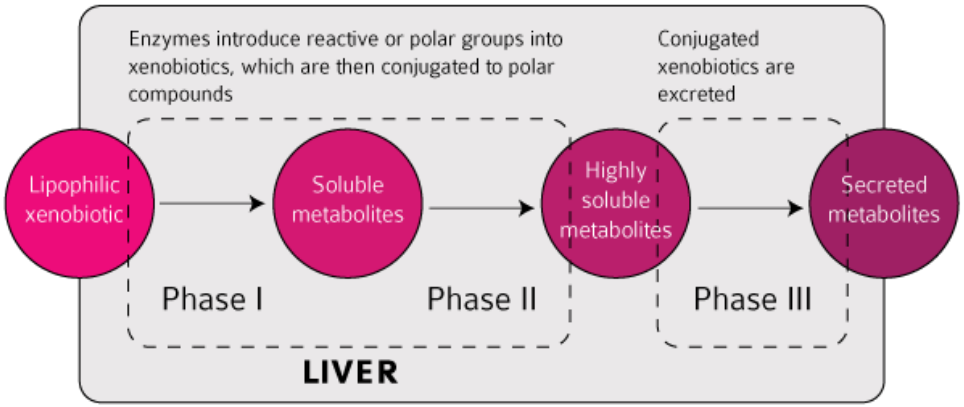
- Reports of serious agitation, psychosis, coma
- Authors report limitations: what is missed?

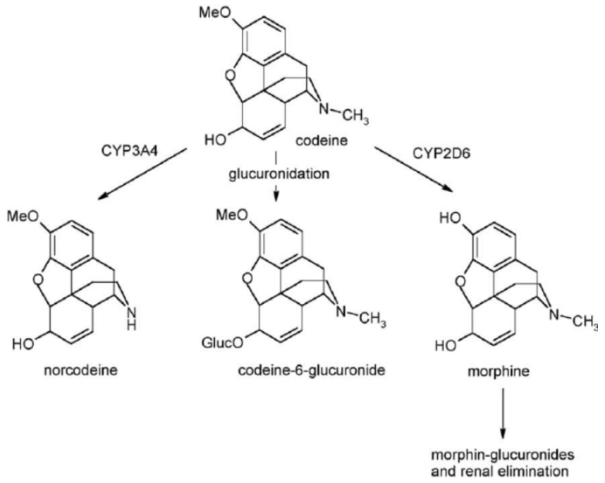
Dines et al., Clinical Toxicology, 2015

NPS are a real challenge for forensic toxicology:

1) Amount of compounds (> 620 monitored in EU and still counting)

2) Unknown metabolic fate of these compounds (Phase-I and Phase-II): which biomarkers to target (parent compound or metabolites)?

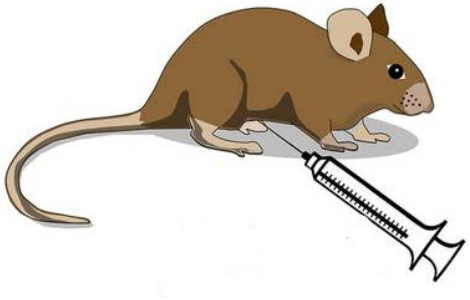


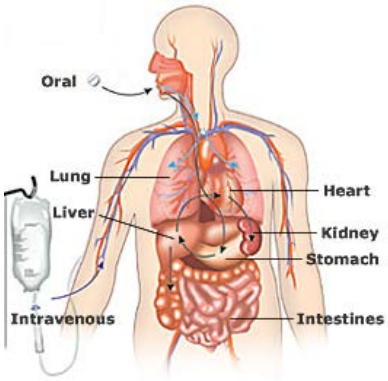


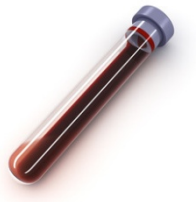
Studying the metabolic fate is highly relevant to identify target biomarkers.

Several strategies possible:

1) In vivo







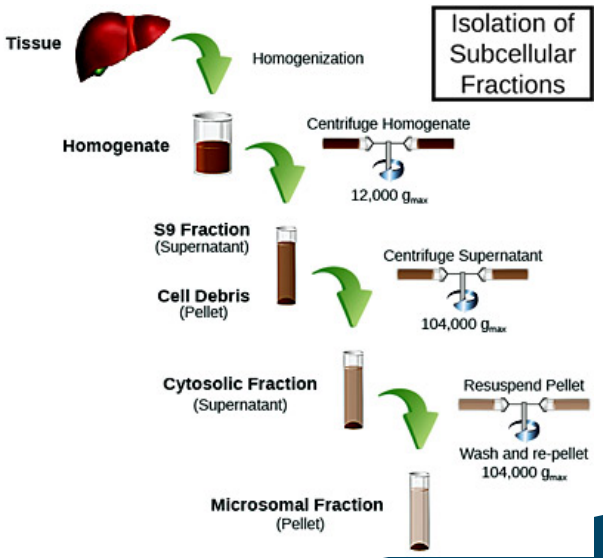
+: closest to reality, complete biological system

-: ethical and safety issues, expensive

Several strategies possible:

2) *In vitro*

- Liver slices, isolated perfused liver (complex)
- Primary hepatocytes
- Liver cell lines
- Human liver S9 fraction
- Human liver subcellular fractions (microsomes, cytosol)



+: easier to control, less expensive

-: representative for *in vivo* biotransformation?

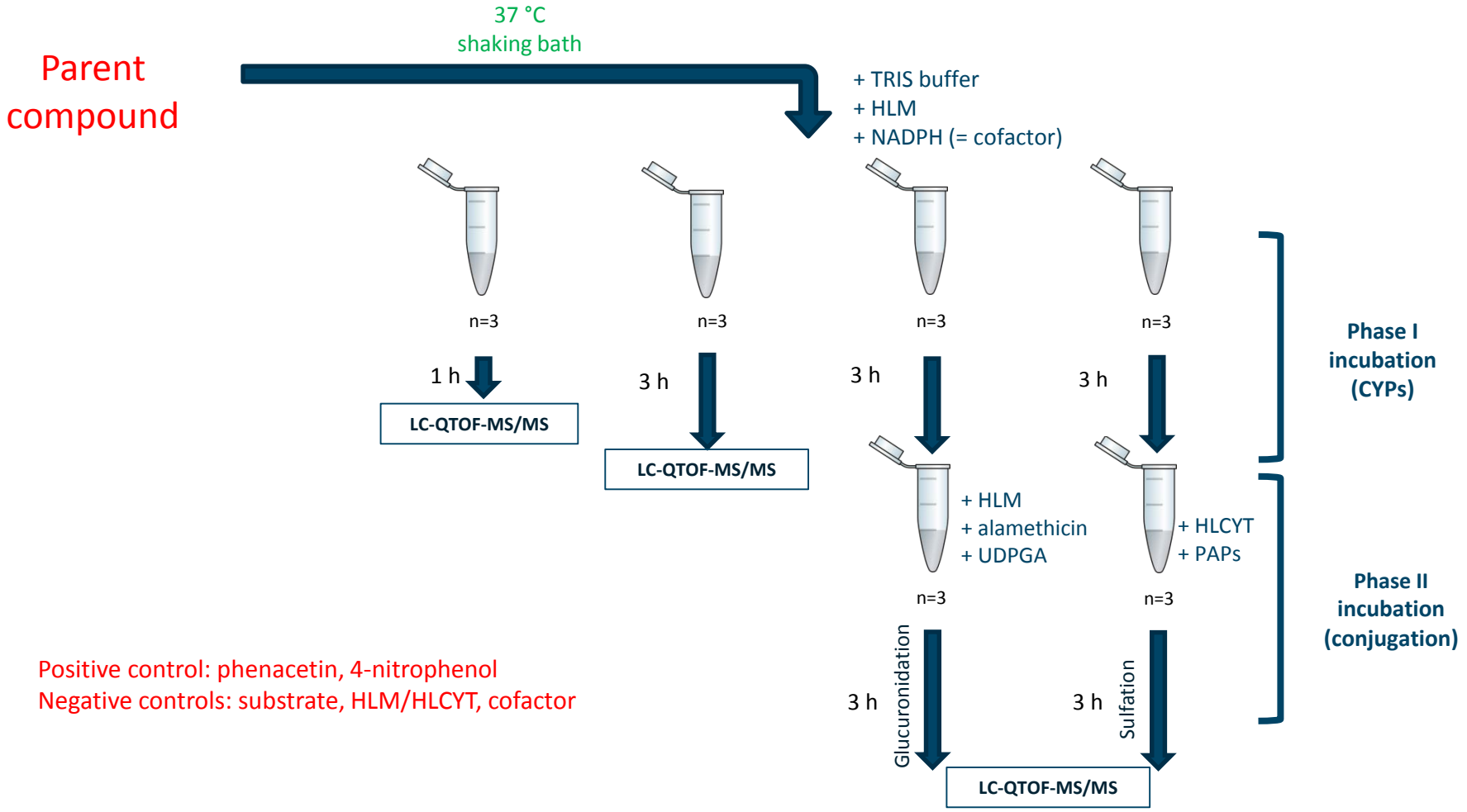
To optimise a straightfoward *in vitro* set-up to elucidate the metabolic pathway of NPS and to identify biomarkers:

- Based on incubations with human subcellular fractions

- Analysis of resulting extracts with liquid chromatography coupled to high resolution mass spectrometry

- Elucidation of metabolites through combination of suspect and non-target data analysis workflows

In vitro incubations experimental setup: straightforward



Positive control: phenacetin, 4-nitrophenol
Negative controls: substrate, HLM/HLCYT, cofactor

HLM = human liver microsomes
HLCYT = human liver cytosol
CYP= cytochrome P450 enzyme system
NADPH = Nicotinamide adenine dinucleotide phosphate
UDPGA = 2,5-uridinediphosphate glucuronic acid
PAPS = adenosine-3-phosphate 5-phosphosulfate

Sample preparation:

- Quenching of the metabolism: + 250 µL ice-cold acetonitrile + 1% formic acid
- Addition of theophylline as ‘internal standard’
- Centrifugation 5 min at 8000 rpm
- Evaporation and reconstitution in 200 µL of a 10/90 (v/v) ACN/Milli-Q water solution

LC-ESI-QTOF-MS

- Agilent 1290 UPLC coupled to Agilent 6530 QTOF
- LC Column: Kinetex C8 (2.1 x 150 mm, 1.7 µm)
- Mobile phase A: MilliQ + 0.04 % FA
- Mobile phase B: 80/20 ACN/Milli-Q + 0.04 % FA
- Run time: 30 minutes (give time to separate!)
- ESI +/-
- Data-dependent acquisition
- Collision energies: 10/20/40 V

Data analysis:

1. Most time-consuming step, but extremely important!
2. Combination of suspect and non-target workflows (complementary)
3. Identification of metabolites: different confidence levels

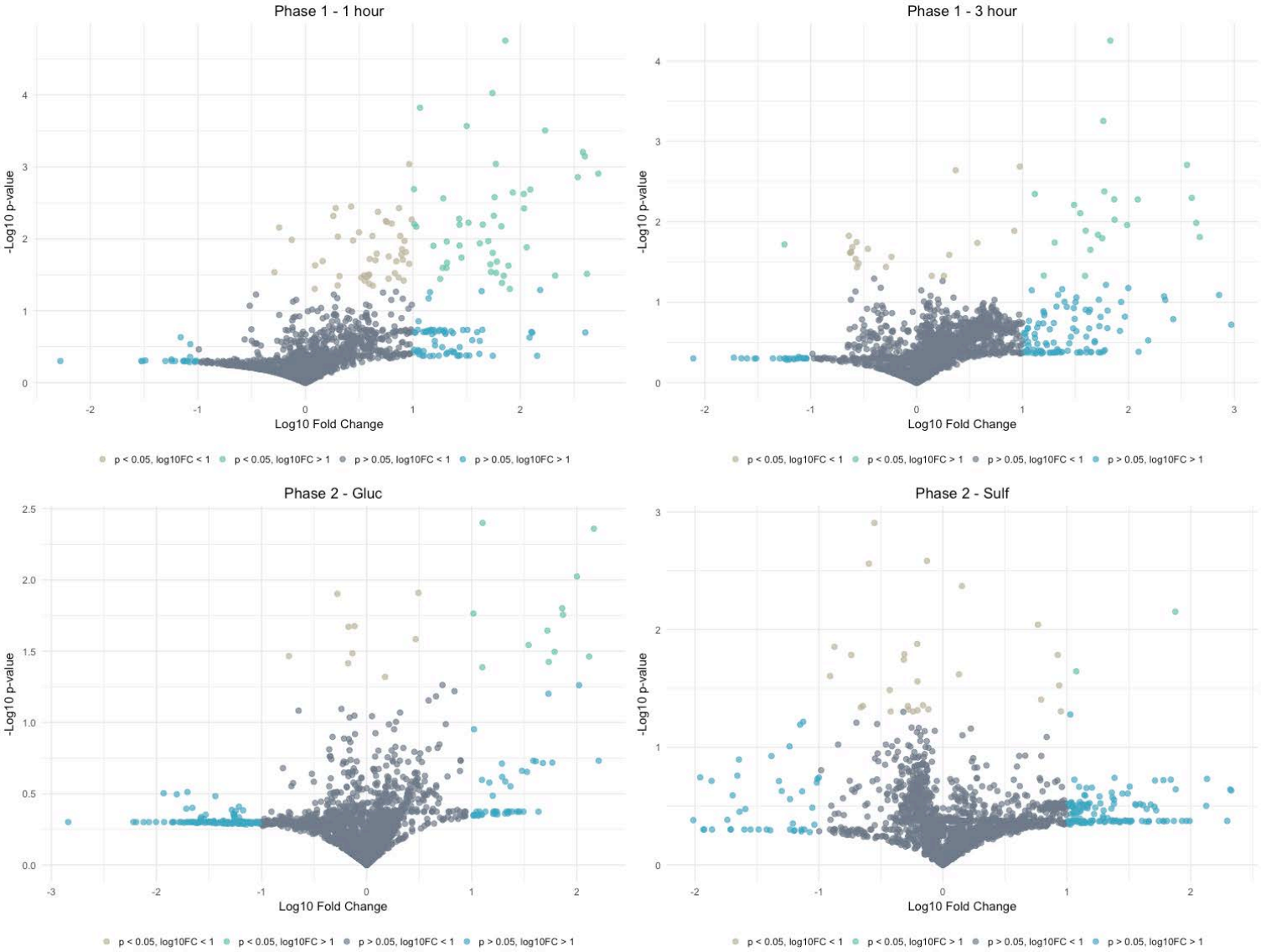
Identification confidence	*Minimum data requirements*
Level 1: Confirmed structure by reference standard	MS, MS^2, RT, Reference Std.
Level 2: Probable structure a) by library spectrum match b) by diagnostic evidence	MS, MS^2, Library MS^2 MS, MS^2, Exp. data
Level 3: Tentative candidate(s) structure, substituent, class	MS, MS^2, Exp. data
Level 4: Unequivocal molecular formula	MS isotope/adduct
Level 5: Exact mass of interest	MS

Schymanski et al., Environ. Sci. Technol., 2014

Suspect screening

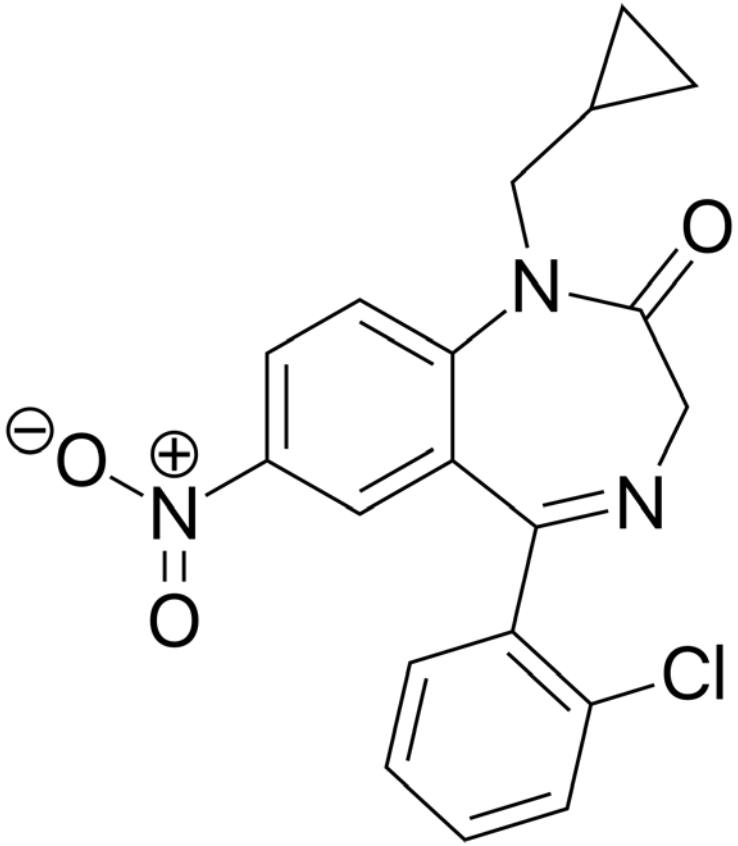
- *In silico* prediction of metabolites with Nexus Meteor (Lhasa Limited) and literature
- Find by Formula algorithm (Agilent MassHunter)
  - Δ m/z < ± 10 ppm
  - Present in 2 out of 3 replicates
  - Not present in negative controls
  - Double bond equivalent (DBE) match
  - Matching isotope pattern

Suspect workflow strategy



Volcano plots

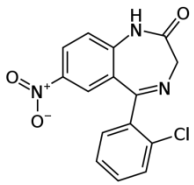
EXAMPLE 1: CLONIPRAZEPAM



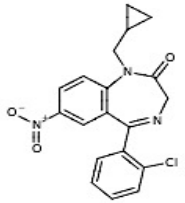
Cloniprazepam = Designer benzodiazepine

- Derived from clonazepam (Rivotril®)

Clonazepam



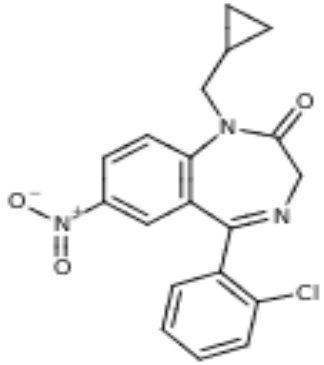
Cloniprazepam



- Sedative, anti-convulsant, muscle relaxant and anxiolytic properties
- Self-medication: alternative to prescription benzodiazepines
- Combination with other drugs

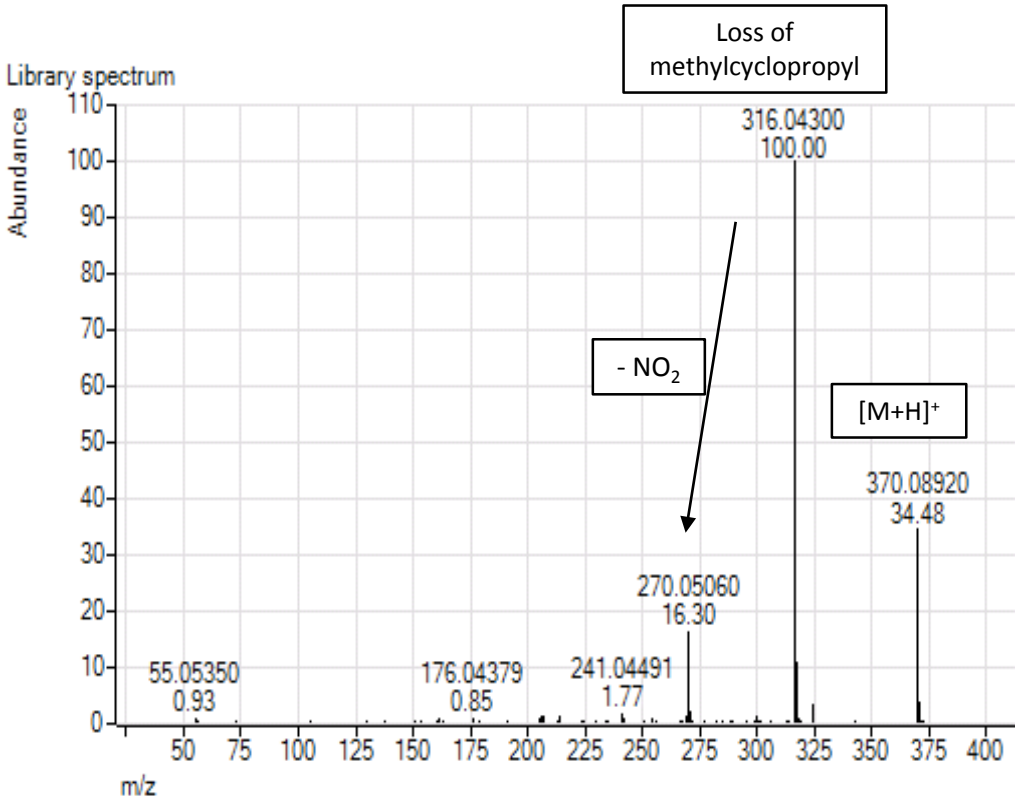
No clinical information available:

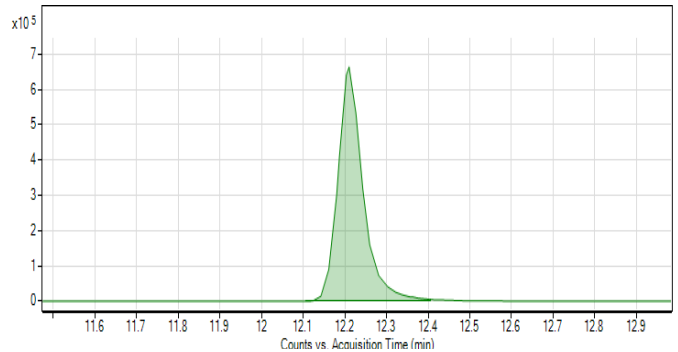
- Pharmacokinetics?
- Metabolism?
- Detection in blood, urine?

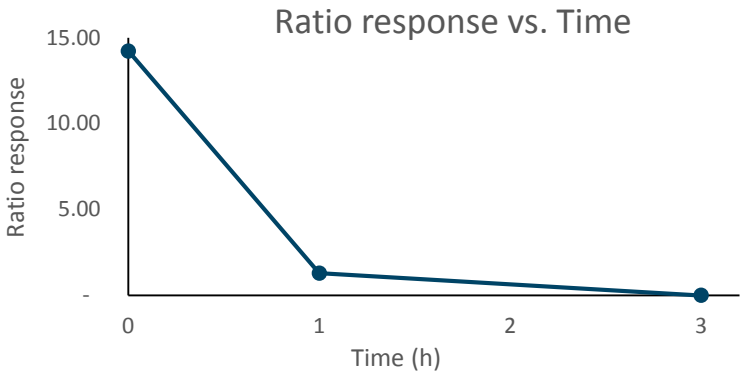


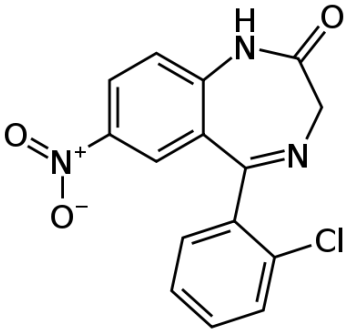
Cloniprazepam/Parent compound

m/z = 370.0953 ([M+H]$^+$)





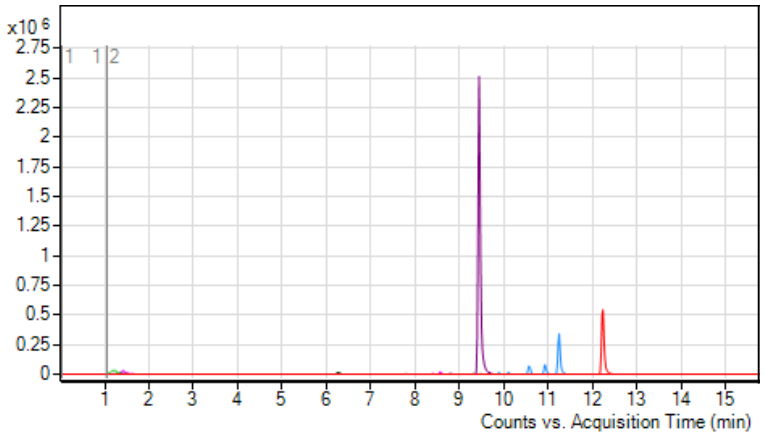


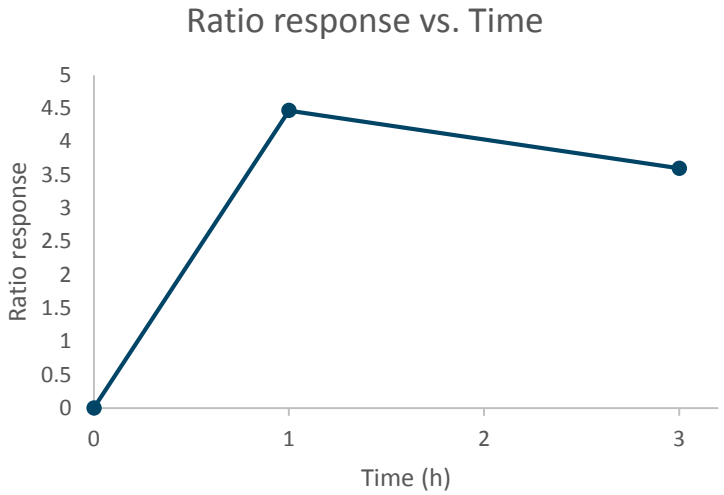


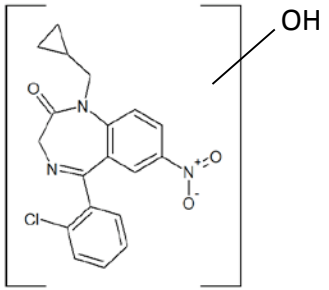
Clonazepam

m/z = 316.0483 ($[M+H]^+$)

- Most prominent metabolite
- Confirmed with MS/MS and analytical reference standard



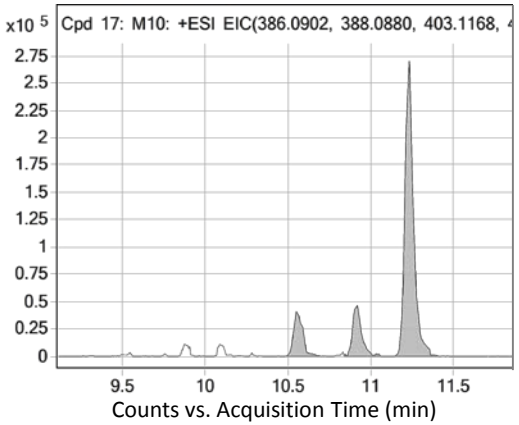




Hydroxy-cloniprazepam

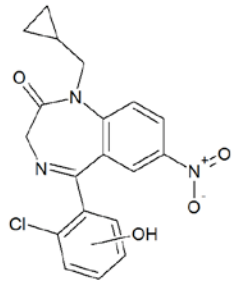
m/z = 386.0902 ($[M+H]^+$)

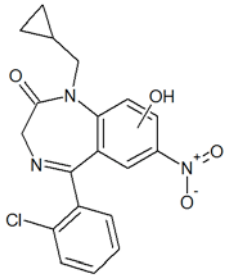
- Different isomers present
- MS/MS available for 3 highlighted peaks
- Fragment *m/z* 332.04 corresponds with hydroxy-clonazepam
  -> no hydroxylation on the methylcyclopropyl side chain

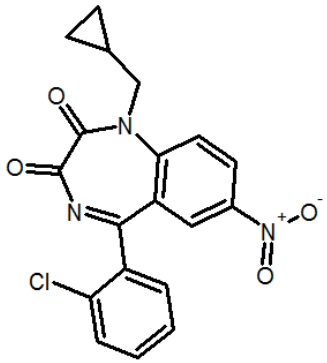


- RT 11.2 min: Loss of H_2O → Ramanathan et al (Anal Chem, 2000, 72: 1352-1359): *"MS/MS data showed loss of water with aliphatic hydroxylation, which was not favoured when the hydroxylation was phenolic"*

- RT 10.6 and 10.9 min: No loss of H_2O present



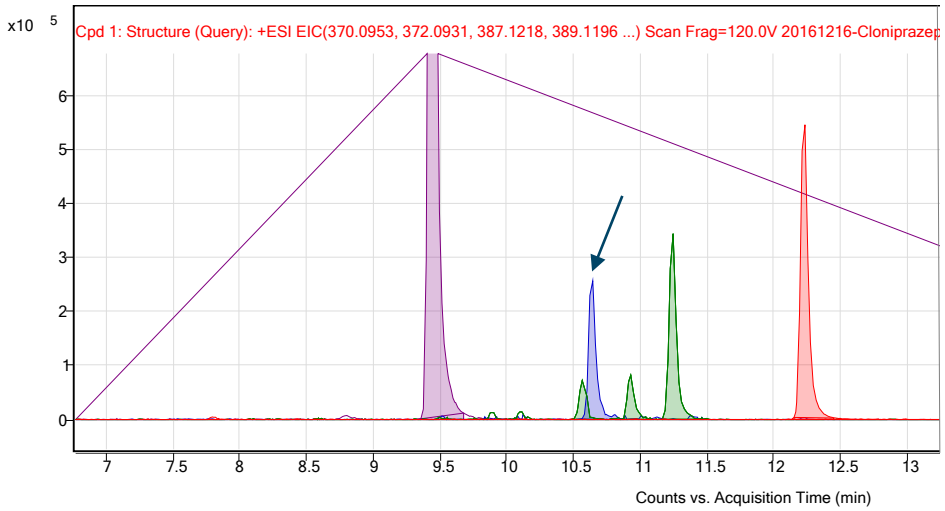




3-keto-cloniprazepam

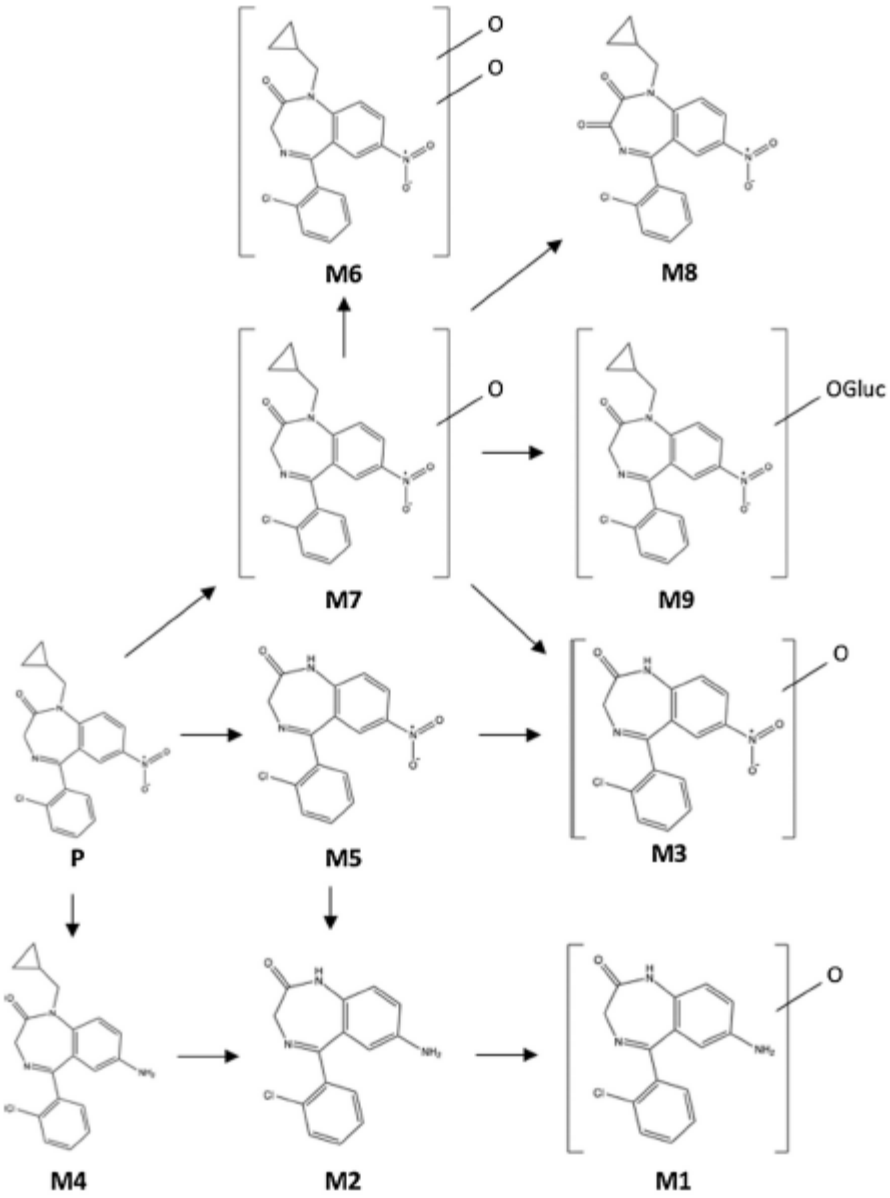
m/z = 384.0746 ($[M+H]^+$)

- Not predicted by *in silico* prediction
- Identified by non-target workflow
- Confirmed by MS/MS and double bond equivalents



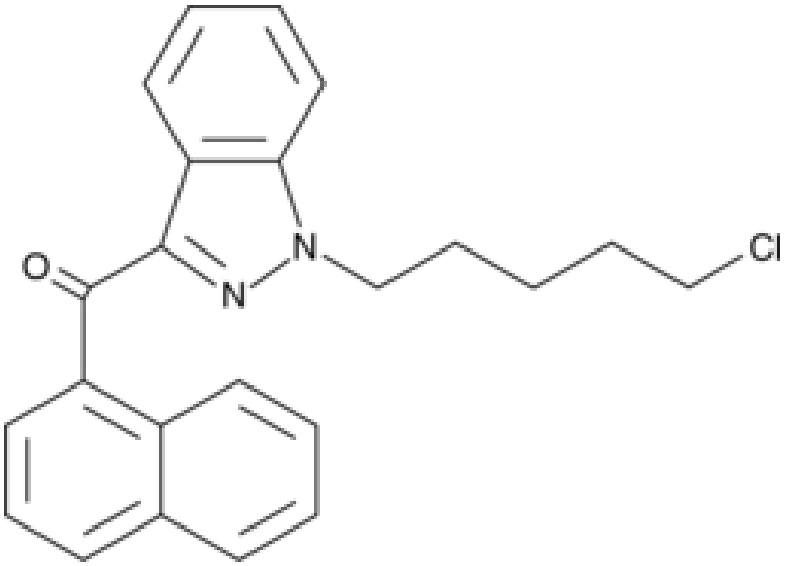
- 9 human *in vitro* metabolites of cloniprazepam identified
  → 8 Phase I and 1 Phase II metabolites
- Clonazepam: most prominent *in vitro* metabolite
- 5 metabolites were specific for cloniprazepam
  - OH-cloniprazepam
  - 7-NH_2-cloniprazepam
  - 3-keto-cloniprazepam
  - Dihydroxy-cloniprazepam
  - Glucuronide of OH-cloniprazepam

  → Possible biomarkers for cloniprazepam intake

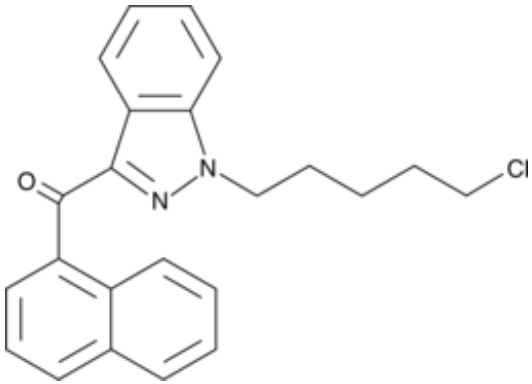


Mortelé et al., J Pharm Biomed Anal, 2018

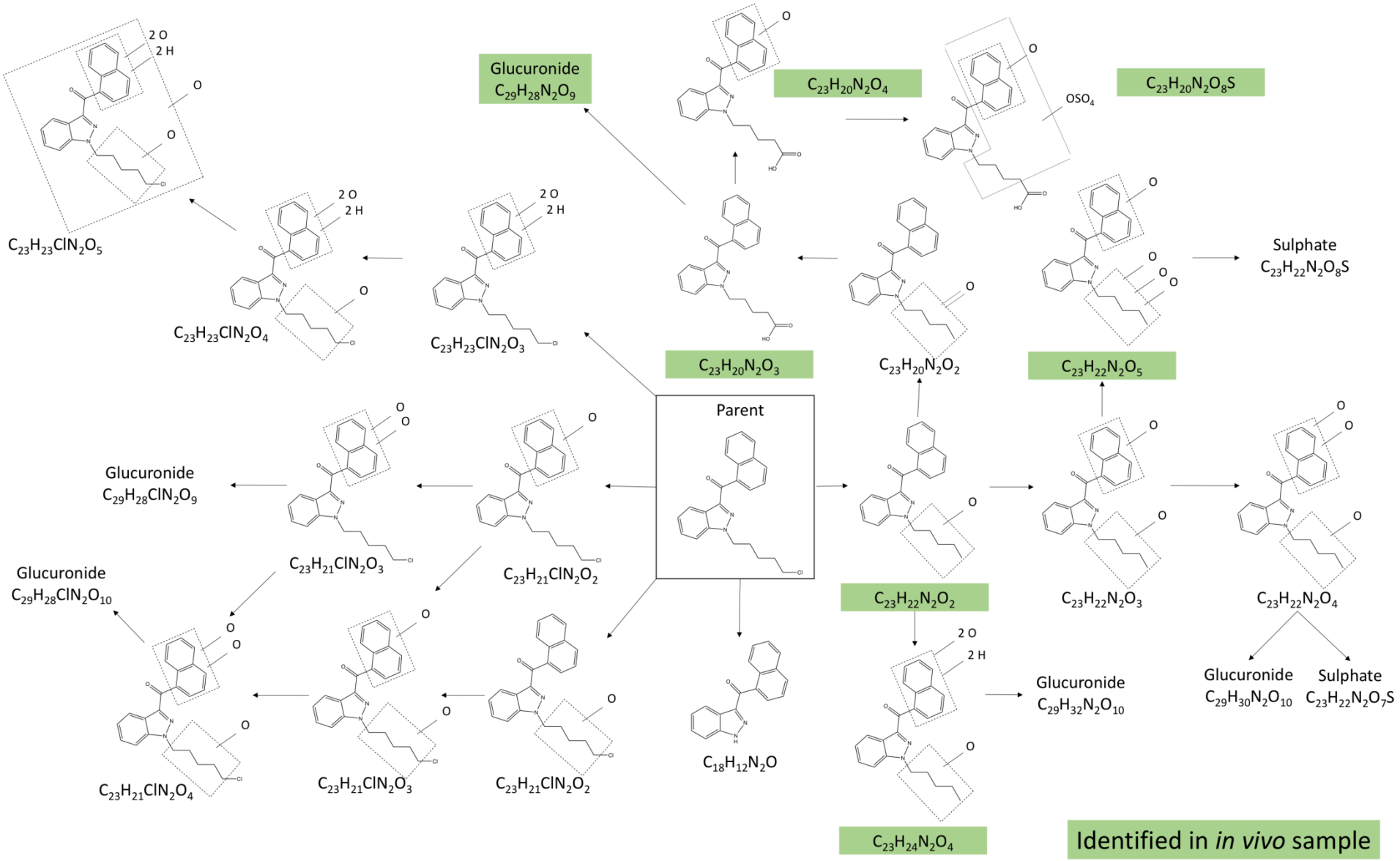
EXAMPLE 2: 5Cl-THJ-018

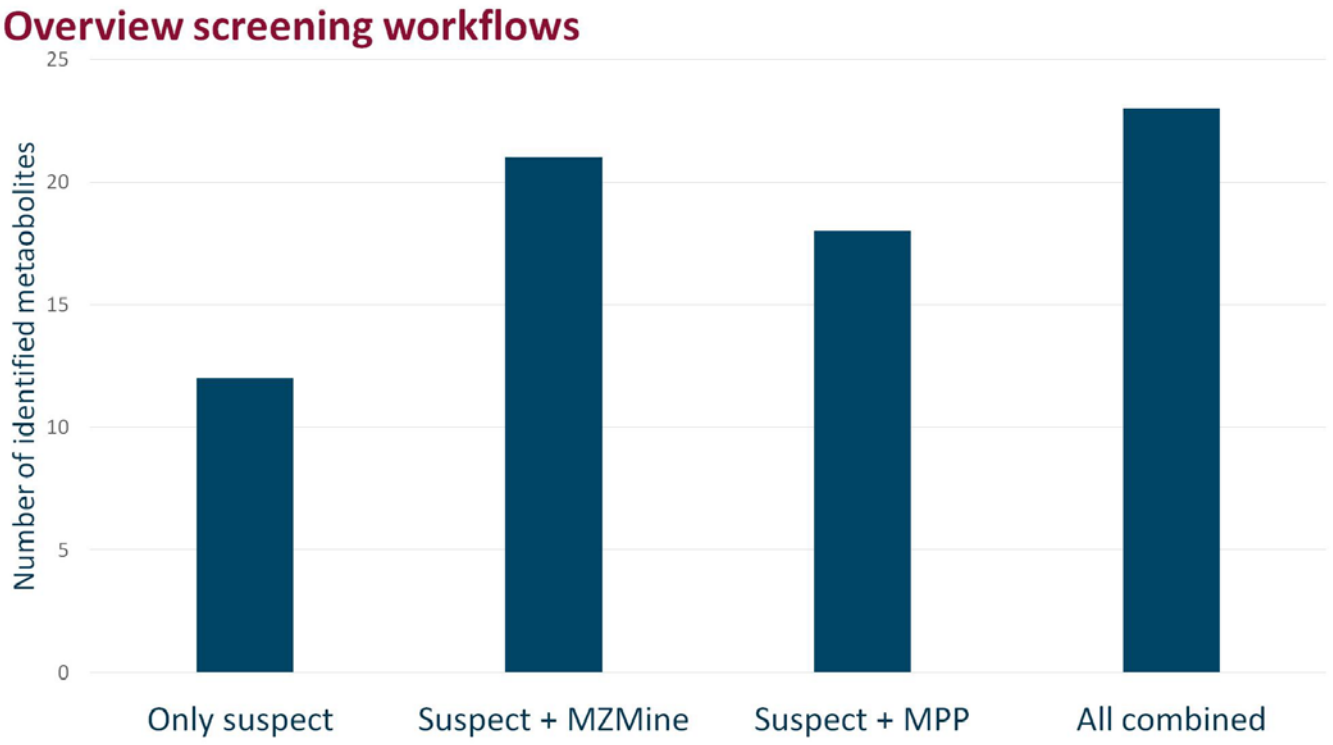


5-Cl-THJ-018 = Synthetic cannabinoid (5-chloropentyl JWH 018 indazole analog)



- Cannabinoid receptor agonist: cannabis-like effects
- The physiological and toxicological properties of this compound have not been determined
- Extensive biotransformation can be expected!
- Possibility to compare the *in vitro* results with *in vivo* case: authentic urine sample from user (who actually thought he used methiopropamine)


Parent
Glucuronide
$C_{29}H_{28}N_2O_9$
$C_{23}H_{20}N_2O_4$
$C_{23}H_{20}N_2O_8S$
$C_{23}H_{23}ClN_2O_5$
$C_{23}H_{23}ClN_2O_4$
$C_{23}H_{23}ClN_2O_3$
$C_{23}H_{20}N_2O_3$
$C_{23}H_{20}N_2O_2$
$C_{23}H_{22}N_2O_5$
Sulphate
$C_{23}H_{22}N_2O_8S$
Glucuronide
$C_{29}H_{28}ClN_2O_9$
$C_{23}H_{21}ClN_2O_3$
$C_{23}H_{21}ClN_2O_2$
$C_{23}H_{22}N_2O_2$
$C_{23}H_{22}N_2O_3$
$C_{23}H_{22}N_2O_4$
Glucuronide
$C_{29}H_{28}ClN_2O_{10}$
$C_{23}H_{21}ClN_2O_4$
$C_{23}H_{21}ClN_2O_3$
$C_{23}H_{21}ClN_2O_2$
$C_{18}H_{12}N_2O$
Glucuronide
$C_{29}H_{32}N_2O_{10}$
Glucuronide
$C_{29}H_{30}N_2O_{10}$
Sulphate
$C_{23}H_{22}N_2O_7S$
$C_{23}H_{24}N_2O_4$
Identified in *in vivo* sample



Complementarity of the workflows!!!!

Conclusions

- Investigation of NPS metabolic fate is necessary to select target biomarkers
- Easy and straightforward set-up with subcellular fractions, but limitations need to be taken into account:
  - Only selected enzymes present (e.g. NAT,…)
  - No complete biological system
  - Qualitative, not quantitative
- Importance of (i) sound data acquisition and (ii) complementary data analysis workflows








Toxicological Centre
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Thank you for your attention! Questions?