Metabolism study of the novel cathinone derivative 3-MMC combining three in vitro approaches, LC-HRMS acquisition and molecular networking tools

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Overview

- New psychoactive substances (NPS) present several analytical challenge for clinical and forensic toxicologists given the need to detect metabolites in urine screening studies. In the absence of human clinical metabolism and pharmacokinetic data alternative in vitro and in vivo models have been considered.
- In this study, three in vitro models of metabolism were used to study 3-methylmethcathinone (3-MMC) in the context of forensic toxicology screening. The models included human liver microsomes, HepaRG cells and HepaRG cells-on-chip (also referred to as organ-on-a-chip). 3-MMC was selected as the NPS candidate molecule and analyzed by high resolution LC-MS/MS.

1. Introduction

Clinical and forensic toxicology, along with related fields such as doping control, require screening methods that can be applied to bio-samples to detect and identify new psychoactive substances (NPS) with high reporting confidence. The challenge, particularly in urine screening, is generating screening methods capable of detecting the NPS parent drug compound and related metabolites. Three in vitro models of metabolism were considered in this study.

- **Human liver microsomes**. Established, simple, cost-effective, results delivered in a short time frame. The model generates activation of specific enzymes with co-factors (CYP, oxygenases, UGT)
  - **Limitations**. Includes inherent variability given the polymorphism of donors, unstable CYP enzyme expression and higher purchase costs.
- **Human hepatoma cell line HepaRG**. Represent immortalized human liver cells derived from a tumor which show comparable gene expression to primary human hepatocytes. They are increasingly applied to NPS screening targets given that they are cost-effective and generate a high number of metabolites.
  - **Limitations**. Low gene expression of CYP2D6, specialized equipment and personnel required for cell cultures. Needs a complex and time-consuming differentiation procedure and only representative of a single donor.
- **HepaRG cells-on-chip (organs-on-a-chip).** A multi-channel 3D microfluidic cell culture chip that mimics the functions and architecture of a human liver. The advantages include strong CYP expression and they closely match physiology compared to cells grown on plates.

2. Materials and Methods

The model NPS compound considered for this study was 3-methylmethcathinone (3-MMC) a commonly encountered psychostimulant. 3-MMC was added to the three in vitro models of metabolism at two concentrations, 5 µM and 50 µM.

- **Human liver microsomes**. 2.5 mg/mL human liver microsomes, 10 mM NADPH; incubation time 60 minutes [3-MMC at 5 µM and 50 µM].
- **human hepatoma cell line HepaRG**. 24 well plate supports (480,000 cells per well), incubation time 48 hours [3-MMC at 5 µM and 50 µM].
- **HepaRG cells-on-chip (organs-on-a-chip).** One channel chip (500,000 cells), incubation time 48 hours [3-MMC at 5 µM and 50 µM].

2.1 LC-MS/MS method

- **LC Separation**.
  - Shim-pack Velox Biphenyl (2.1x100mm 2.7µm); flow rate 0.3 mL/min.
  - Binary gradient; water + 2 mM ammonium formate 0.002 % formic acid, and methanol + 2 mM ammonium formate 0.002 % formic acid.
- **Mass Spectrometry Detection**. QTOF LCMS-9030 using external mass calibration
  - MS mass scan m/z 70-1000; 100 msecs; positive ion mode.
  - DDA-MS/MS mass scans m/z 40-1000; collision energy spread 5-55V.
  - DDA-MS/MS; 20 dependent MS/MS mass scans; 25 msecs for each mass scan.
  - Cycle time less than 0.55 second for MS and MS/MS mass scans.
- **Data processing**.
  - **LabSolutions Insight Analyze.** Component detection:
    - Default parameters for peak detection were applied (spectral intensity 300, response threshold set to very low).
    - A targeted screening list of biotransformations was applied (accurate mass and formulae for metabolites including frequently observed mass differences and formulae reported in literature for metabolite identification projects). An accurate mass match criteria was set to ±5 ppm.
  - **LabSolutions Insight Assign.** Verify metabolite identification:
    - Assign algorithm was applied to verify fragment ion MS/MS spectra for each metabolite to support metabolite identification.
  - **Molecular networking and Global Natural Product Search (GNPS).** Aim: to increase the search for metabolite identification and discovery.
    - Data analysis using MS-DIAL followed by GNPS.
    - Identify potential similarities among all MS/MS spectra within the dataset and to propagate annotation to unknown but related molecules.

3. Results

3.1 Metabolite detection and annotation

LabSolutions Insight Analyze application was used to locate ions that behave as recognized chromatographic features (ion intensities rise and fall in abundance in a covariant manner).

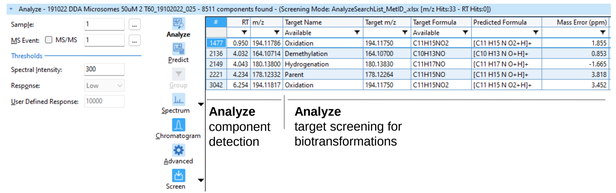


Figure 1. The Analyze component detection algorithm was applied to LCMS analysis of metabolites in each of the three in vitro models of metabolism. Default parameters for peak detection were applied with a spectral threshold of 300. A biotransformation search list was applied to annotate detected metabolites (the match criteria included an accurate mass value within ±5 ppm and predicted formula).

3.2 Metabolite identification

DDA-MS/MS was used to increase reporting confidence in metabolite identification. Precursor ions agreed with the expected parent and metabolite biotransformation products with the DDA-MS/MS also consistent with previously reported literature.

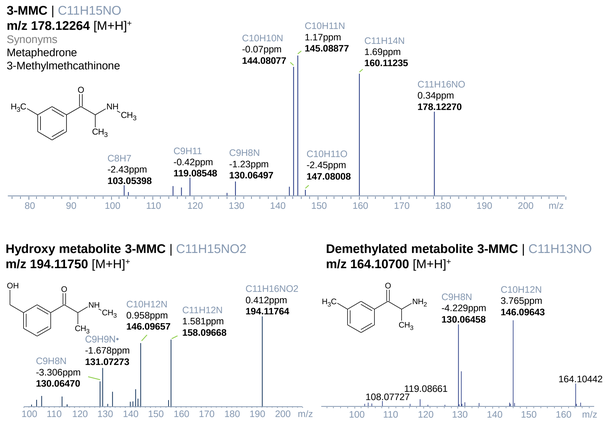


Figure 2. DDA-MS/MS spectrum for the parent 3-MMC, hydroxy and demethylated metabolites using a collision energy spread of 5-55 V. Assign was used for fragment ion annotation.

3.2 Other approaches in NPS metabolite detection

GNPS was applied to support the identification of unknown NPS compounds. MS and DDA-MS/MS data were processed using GNPS using default parameters.

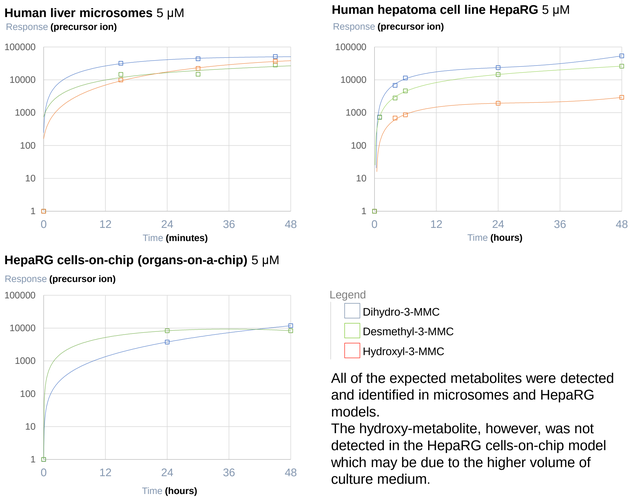
- **GNPS Molecular networking.** Detected the following metabolites:
  - Hydroxy metabolite 3-MMC (C11H15NO2 m/z 194.11750 [M+H]+)
  - Demethylated metabolite 3-MMC (C11H13NO m/z 164.10700 [M+H]+)

3.3 Influence of in-vitro metabolite model

Key findings:

- **Human liver microsomes and Human hepatoma cell line HepaRG**. Both models resulted in the detection of the 3-MMC parent molecule and three metabolites; hydroxy metabolite, demethylated and hydrogenated 3-MMC. The time course to generate all three metabolites was far quicker using human liver microsomes.
- **HepaRG cells-on-chip (organs-on-a-chip).** In this study, the multi-channel 3D microfluidic cell culture chip generated 3-MMC parent molecule and two metabolites; demethylated and hydrogenated 3-MMC. The hydroxy metabolite was not detected.

3.4 In-vitro NPS response-time profile



All of the expected metabolites were detected and identified in microsomes and HepaRG models.
The hydroxy-metabolite, however, was not detected in the HepaRG cells-on-chip model which may be due to the higher volume of culture medium.

Figure 3. Response-time profiles for the three in-vitro models of metabolism.

4. Conclusions

- Three in vitro incubations were used in this study presenting specific advantages and limitations.
  - Human liver microsomes provided a relatively simple methodology and delivered results quickly at low cost. Microsomes are one of the best characterized in vitro models for drug metabolism research, however, not all enzymes are expressed which may be a confounding factor when relating to human in vivo metabolism.
  - HepaRG cells have the potential to generate a high number of metabolites and show gene expression levels for metabolizing enzymes comparable to primary human hepatocytes. The cell model also provides an indication of drug kinetics. The limitations are the need for specialized equipment and staff.
- The study generated expected metabolites (using a screening list of precursor biotransformations and DDA-MS/MS correspondence with published literature). Molecular networking tools provided an additional workflow to help metabolite discovery.

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