ISSUE 1

# Cayman NPS Metabolism Monograph

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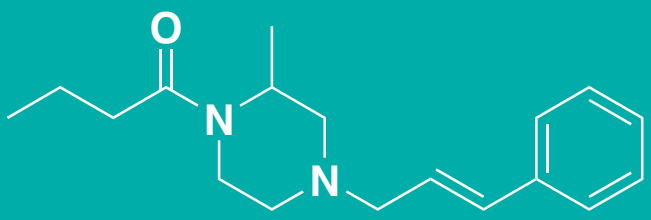
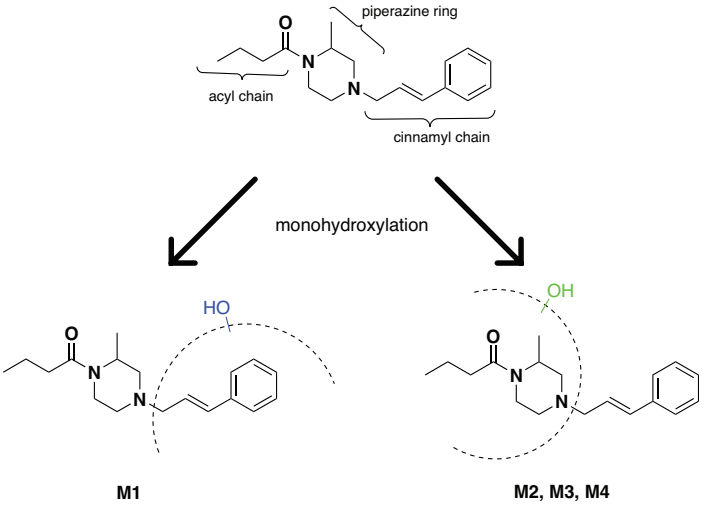
## 2-methyl AP-237

Item No. 26485

Synthetic Opioid Analgesic



2-methyl AP-237 was originally synthesized and reported in Japan in the 1970s.[1] Related analogs have been used for pain management internationally but never in the US. This compound has appeared on the dark web and illicit research chemical websites, and it has been identified by forensic chemists and toxicologists in seized samples and drug screens. The goal of this monograph is to identify the probable metabolites of this newly emerging synthetic opioid using human liver microsome (HLM) assays and computational simulations. By using high-resolution Orbitrap mass spectrometry, we aim to provide forensic toxicologists with the key mass spectrometry fragments and ions needed to identify the novel synthetic opioid, 2-methyl AP-237.




Observed phase I metabolites of 2-methyl AP-237 detected using high mass accuracy fragments generated by LC-MS/MS.

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## Mass Spectrum of 2-methyl AP-237

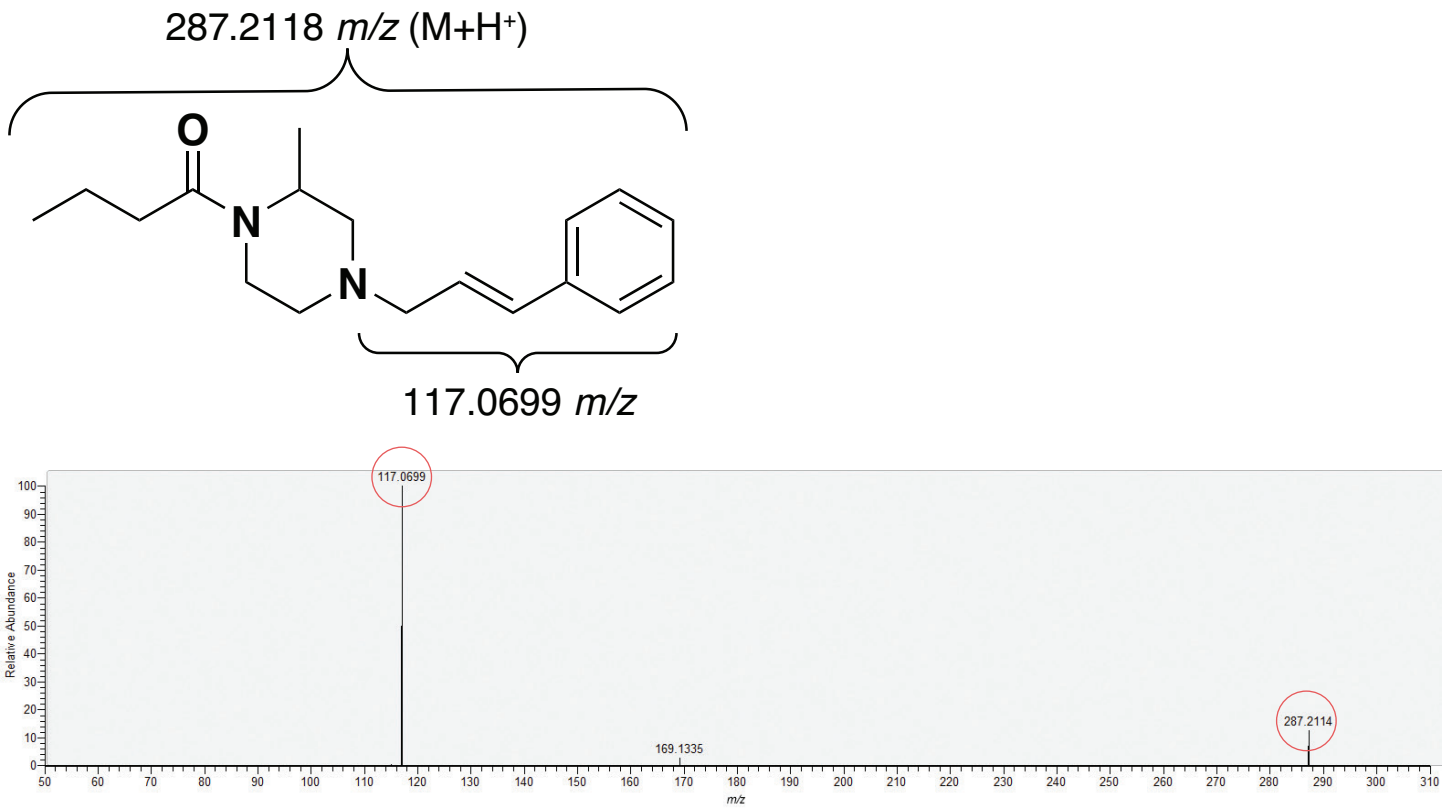



**Figure 1.** Structure and high-resolution mass spectrum of the parent compound 2-methyl AP-237.

## 2-methyl AP-237 Metabolite Formation and Detection

The parent compound 2-methyl AP-237 was incubated with HLM to form phase I metabolites. An aliquot from the incubation was injected onto the Ultra-High Performance Liquid Chromatograph (UHPLC) and then fragments of eluted metabolites were detected using a high-resolution Orbitrap mass spectrometer. The total ion chromatogram (TIC) along with extracted ion chromatograms (EIC) were used to identify expected metabolites that formed from HLM incubation with 2-methyl AP-237 (**Figure 2**). The TIC (in black) shows potential metabolites at 4.79 min and 5.62 min retention times (RT), as well as the parent, 2-methyl AP-237, at 5.42 min. Based on the EIC (in red), four main metabolites eluted at retention times of 4.67 (M1), 4.80 (M2), 5.22 (M3), and 5.62 min (M4). The stacked chromatograms show the TIC in black (representing both parent compound and metabolites) and the EIC in red (showing the four main phase I metabolites formed from HLM incubation). All four phase I metabolites displayed a molecular ion mass of 303.2067 *m/z* (± 3 ppm), which is indicative of monohydroxylation.

It should be noted that after one hour of microsomal incubation and prior to addition of NADPH cofactor, a minor peak appears at 4.59 min. This peak had the same fragmentation profile and relative ion intensities as 2-methyl AP-237, which seems to indicate that some minor cofactor-independent transformation or isomerization took place.

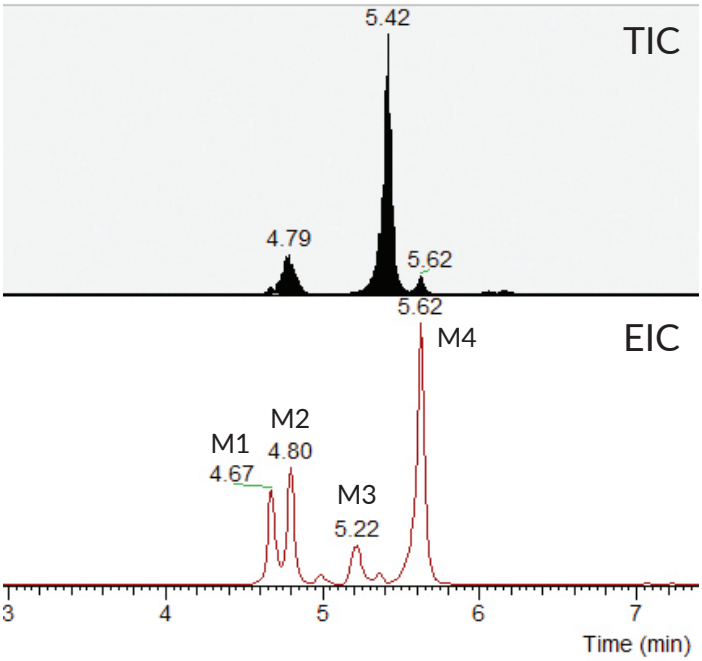



**Figure 2.** TIC (top, black) and EIC (bottom, red) of 2-methyl AP-237 post-incubation.

## Metabolite 1 (M1)

The first eluting metabolite (4.67 min) yielded the mass spectrum below with major fragments of 133.0647 *m/z* and 171.1491 *m/z*. The presence of the unmodified acyl/piperazine fragment at 171.1491 *m/z* and the presence of the hydroxylated cinnamyl chain seems to help narrow down the location of oxidation. Instead, monohydroxylation appears to have occurred on the cinnamyl tail chain at the allylic position or on the benzene ring (133.0647 *m/z*).

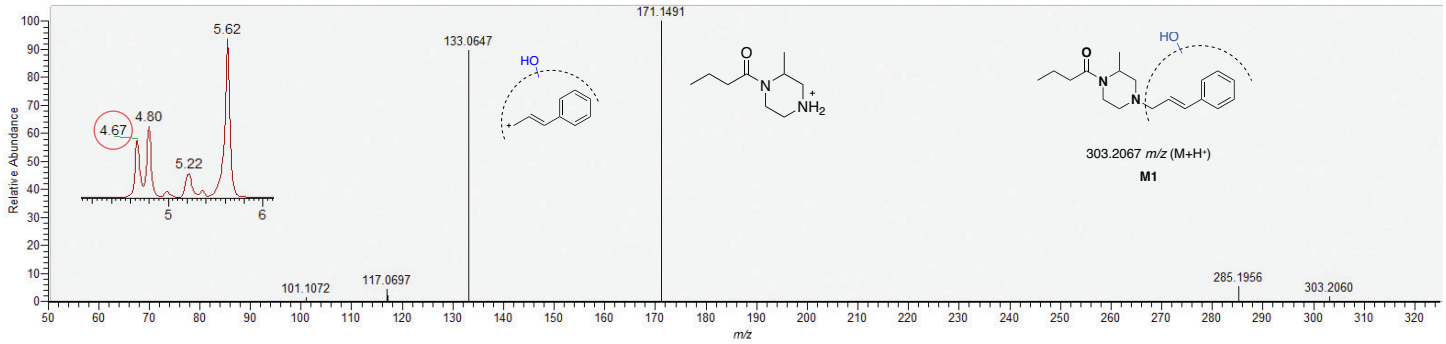
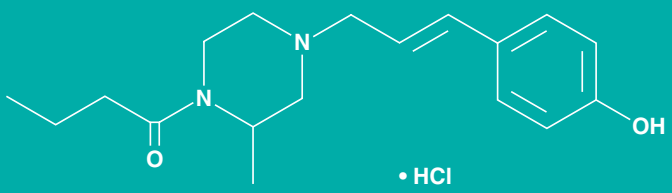



**Figure 3.** EIC and high-resolution mass spectrum of M1.

## Potential Metabolite of 2-methyl AP-237

*para*-hydroxy 2-methyl AP-237 (hydrochloride)
Item No. 34456

Suspected as the first eluting metabolite of 2-methyl AP-237 based on data presented in this monograph



## Metabolite 2, 3, and 4 (M2, M3, and M4)

The second, third, and fourth eluting metabolites had retention times of 4.80 min, 5.22 min, and 5.62 min, respectively. All three metabolites yielded the same fragments of 117.0698 *m/z* (or 117.0699 *m/z*) and 303.2061 *m/z* (or 303.2062 *m/z*), indicating that monohydroxylation took place on the piperazine ring or acyl chain instead of the cinnamyl chain. Only **M2** yielded an additional fragment of 185.1285 *m/z*, giving further indication that the position of hydroxylation is on the acyl chain or piperazine ring. Metabolite **M4** appears to be the major metabolite based on the EIC (in red).

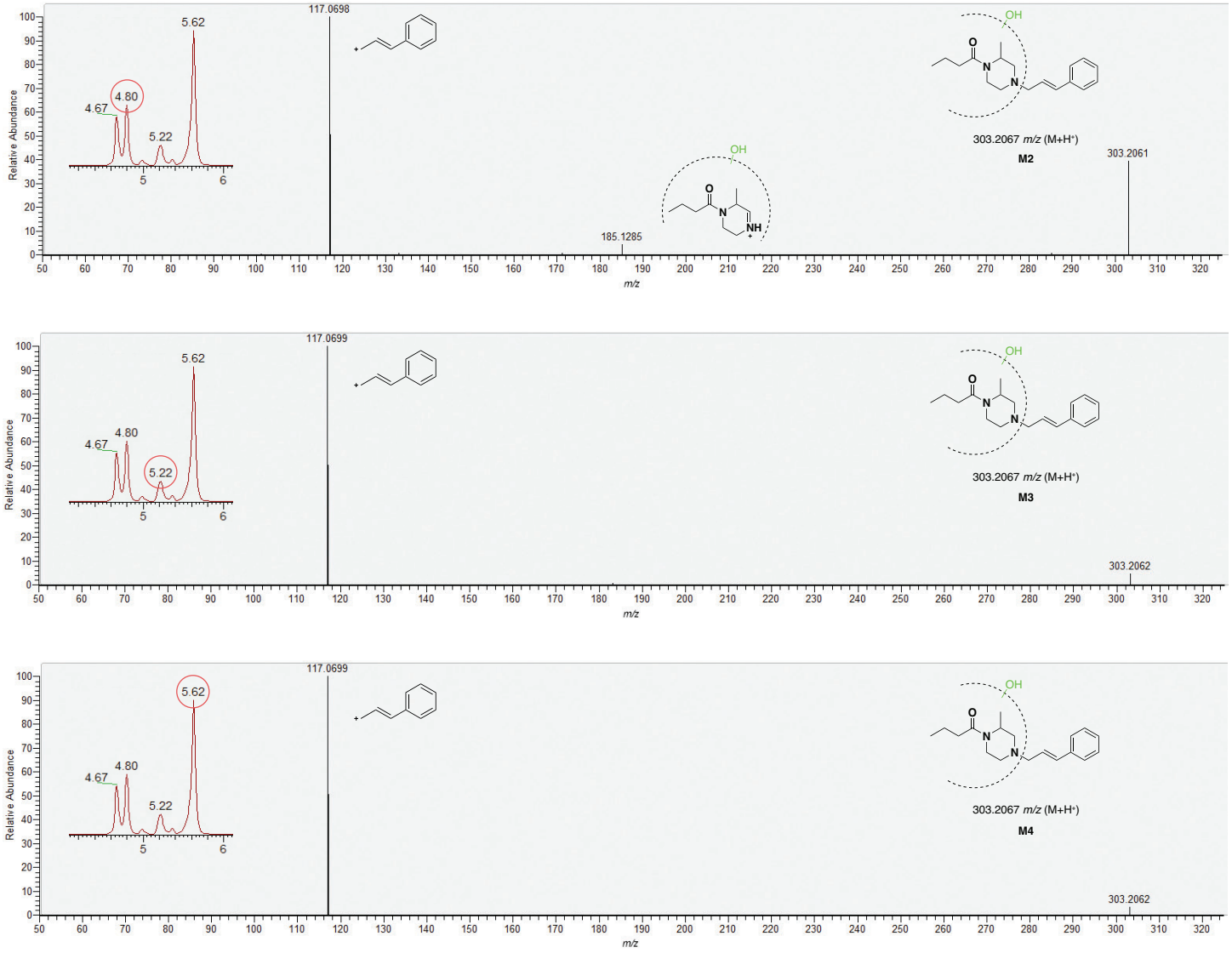
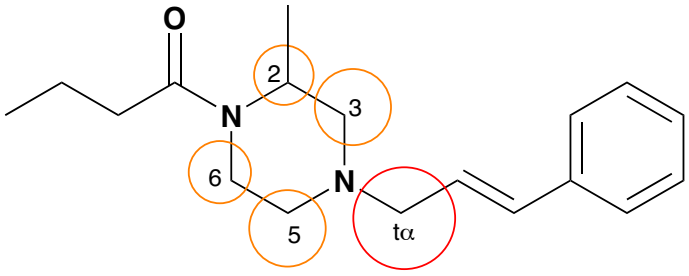



**Figure 4.** EIC and high-resolution mass spectrum of M2, M3, and M4.

The lack of characterisitic fragment ions in the MS/MS data for M2-M4 prevented us from narrowing the structures down further. Therefore, we turned to a metabolism QSAR model and molecular dynamics to help predict more specifically where monohydroxylation was likely to occur.

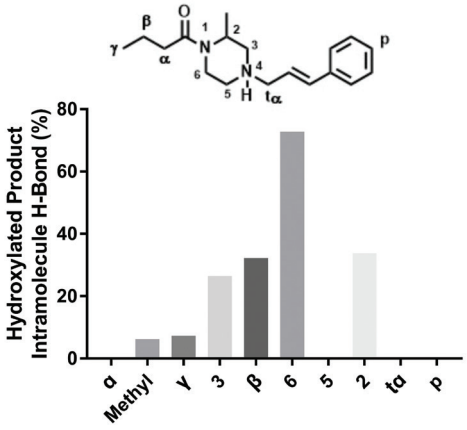
## Site of Metabolism Prediction (QSAR Model)

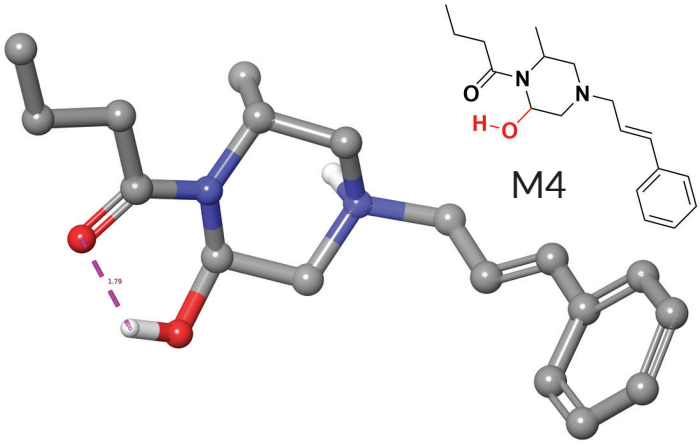
A quantitative structure activity relationship (QSAR) model was used to predict the intrinsic reactivity of 2-methyl AP-237 to metabolism by CYP3A4. The Schrödinger QSAR model is based on the known reactivity of 384 small molecules and scores empirically by evaluating reactivity of specific functional groups.[2] This model helps to identify which sites on 2-methyl AP-237 will potentially be hydroxylated during phase I metabolism. The most probable sites for metabolic oxidation were found to be the allylic position (tα) illustrated with a red circle and the piperazine ring positions (2, 3, 5, and 6) illustrated with orange circles. The size of the circles indicates differences in probability of oxidation, with tα having the highest probability. As stated previously, the first eluting metabolite **M1** (4.67 min) yielded a mass spectrum with fragments of 133.0647 *m/z* and 171.1491 *m/z*. Based on the QSAR model, hydroxylation at the allylic position (tα) is the most probable oxidative site. Metabolites **M2**, **M3**, and **M4** all had fragments of 117.0698 *m/z* (or 117.0699 *m/z*) and 303.2061 *m/z* (or 303.2062 *m/z*), which indicated oxidation at either the acyl chain or piperazine ring. Between the acyl chain and piperazine ring, the QSAR model predicted the most probable sites for oxidation were on the piperazine carbons.




## Intramolecular Hydrogen Bonding Potential (Molecular Dynamics)

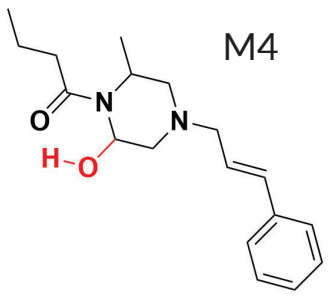
Surprisingly, the primary metabolite **M4** displayed an increased hydrophobic footprint relative to the parent molecule based on a longer retention time (5.62 min). A molecular dynamics simulation within a water solvent model was used to predict the probability of intramolecular H-bonding for hydroxylated metabolites at positions on the piperazine ring and on acyl chain positions (α, β, γ).[3] A molecular dynamics simulation is an explicitly stated computational experiment for studying the physical movements of atoms and molecules over time. It was postulated that intramolecular hydrogen bonding may reduce the polarity of one of the unknown hydroxylated metabolites relative to the parent 2-methyl AP-237. In addition to being one of the sites predicted to be oxidized by the QSAR model, monohydroxylation at the 6 position is most probable for having an intramolecular H-bond which would make the compound overall less polar in solution than 2-methyl AP-237.



## Conclusion

Incubation of 2-methyl AP-237 with HLM resulted in four monohydroxylated phase I metabolites (**M1**, **M2**, **M3**, and **M4**). Based on MS/MS fragmentation data, we can conclude that one of the metabolites is hydroxylated on the cinnamyl chain region and the other three are hydroxylated on either the acyl chain or the piperazine ring. Using QSAR predictions, followed by molecular dynamics simulations, we can further narrow down that the most likely site for hydroxylation on the piperazine ring is carbon C6 with C6-OH (**M4**) being the predicted major metabolite.




## Methodology and Instrumentation

*In vitro* metabolism studies

The compound 2-methyl AP-237 was diluted to necessary concentration by preparing a 50 mM stock DMSO solution and further diluting with phosphate buffer (pH 7.4). The compound solution was then incubated for one hour at 37°C, with HLM and the added cofactor NADPH. At one hour the reaction was quenched with acetonitrile, spun down on a centrifuge, and an aliquot of the mixture was injected onto the UHPLC. Mass fragments were detected using an Orbitrap mass spectrometer.

- Microsomes: 50-pool mixed-gender human liver microsomes, 20 mg/ml protein conc. (Sekisui XenoTech, H0610)
- Analyte: 2-methyl AP-237 (hydrochloride) (Cayman Chemical, Item No. 26485)
- Instrumentation and analysis LC-MS/MS:
  - Dionex™ UltiMate™ 3000 UHPLC (Thermo Scientific™)
  - Acquity UPLC™ BEH C8 column, 1.7 µm, length 2.1 x 100 mm (Waters™)
  - Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific™)
  - Xcalibur™ software v. 4.0 (Thermo Scientific™)

Computational Studies

- QSAR CYP3A4 intrinsic reactivity model. (**Schrödinger Release 2018-3**: P450 Site of Metabolism, Schrödinger, LLC, New York, NY, 2018)
- 10 ns molecular dynamics simulation performed within explicit water solvent model. (**Schrödinger Release 2018-3**: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2018. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2018)

### References


1. Nishimura, N., Kiuchi, M., Kanetake, Y., *et al.* Clinical evaluation of a new analgesic agent Ap-237. *Masui* **19(6)**, 653-656 (1970).
2. Feng, S. and Friesner, R.A. Prediction of cytochrome P450-mediated metabolism using a combination of QSAR derived reactivity and induced fit docking. *arXiv q-bio* **1811.09366**, (2018).
3. Bowers, K.J., Chow, D.E., Xu, H., *et al.* Scalable algorithms for molecular dynamics simulations on commodity clusters. *Proceedings of the 2006 ACM/IEEE Conference on Supercomputing* (2006).

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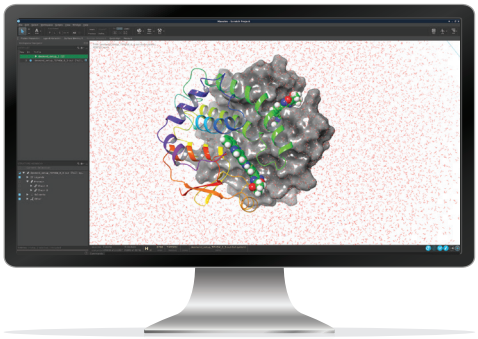
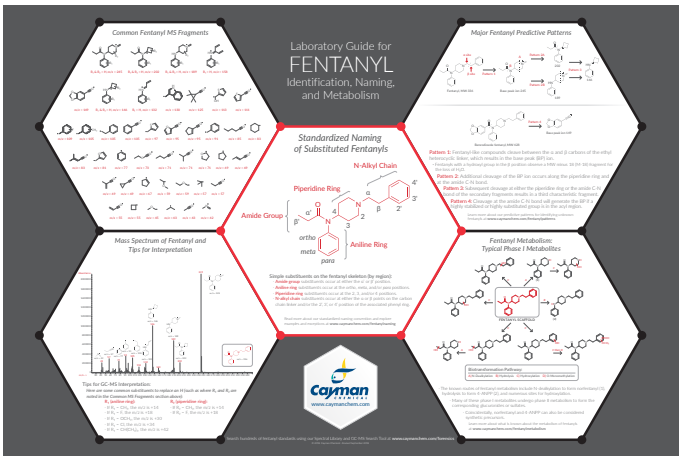
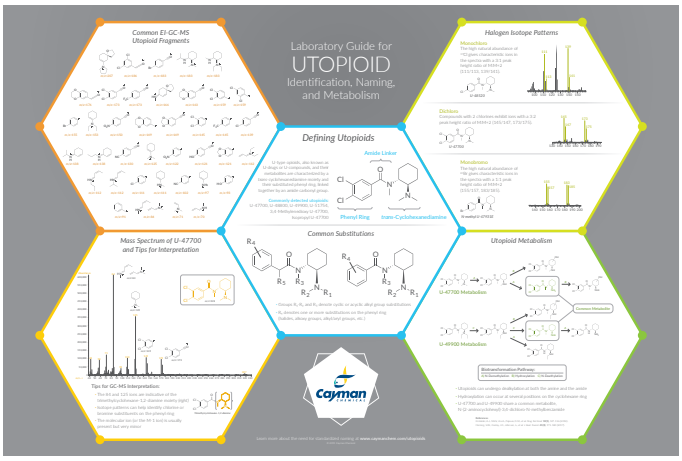


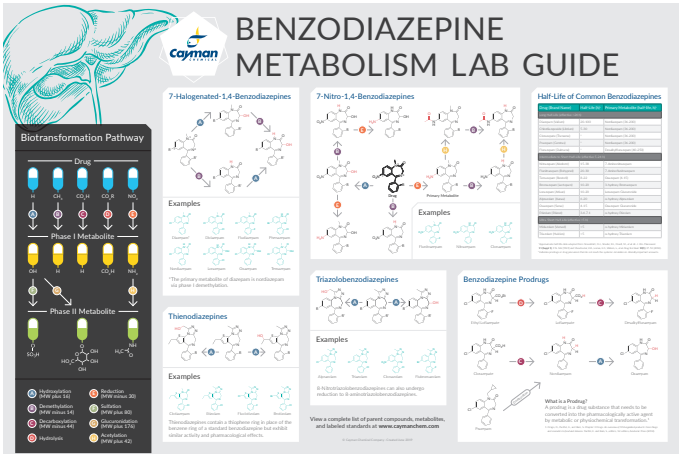
# Drug ID, Naming, & Metabolism Posters



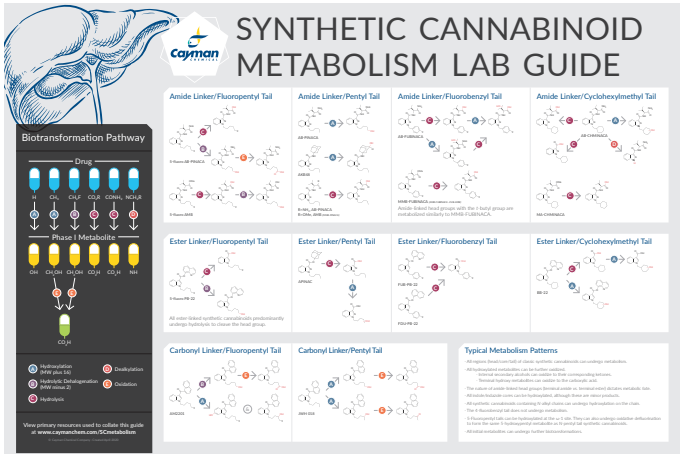

Fentanyl Lab Guide




Utopioid Lab Guide




Benzodiazepine Metabolism Lab Guide




Synthetic Cannabinoid Metabolism Lab Guide

Includes common MS fragments, tips for interpretation of mass spectrums, major predictive patterns, and typical phase I and/or phase II metabolites.

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