cfsre The Center for Forensic Science Research & Education

Evaluating Cross Reactivity of New Psychoactive Substances (NPS) on Immunoassay in Whole Blood

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SOFT 2022 Annual Meeting

Disclaimer

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NIJ | National Institute of Justice

STRENGTHEN SCIENCE. ADVANCE JUSTICE.

National Safety Council's Alcohol, Drugs and Impairment Division (NSC-ADID)

- Started an initiative to standardize testing practices in toxicology labs for DUID cases and improve the quality of data surrounding DUID
- Surveyed labs on their testing practices, resources, various technologies, etc.
- NSC-ADID started issuing these recommendations in 2007 and released a recent update in 2021

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Alcohol, Drugs & Impairment Division

NSC-ADID Survey

- From the 2020 survey of 65 labs, the most common screening methods were:
  - 51% - Enzyme-Linked Immunosorbent Assay (ELISA)
  - 35% - Gas Chromatography Mass Spectrometry
  - 31% - Liquid Chromatography Tandem Mass Spectrometry
  - 23% - Liquid Chromatography High Resolution Mass Spectrometry

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Pros and Cons of Immunoassay

Pros

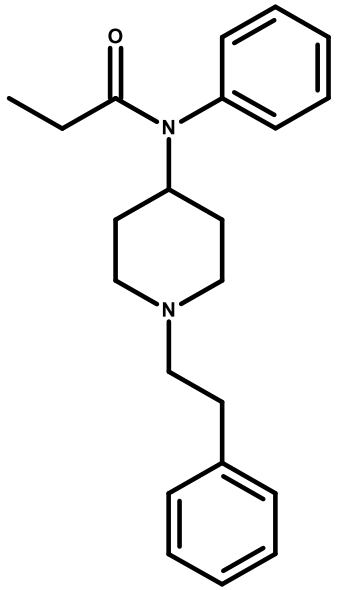
1. Sensitivity
2. Easy to automate
3. Long shelf life
4. Exclude drug prior to confirmation
5. Fast and simple
6. Commercially available
7. Easily implemented

Cons

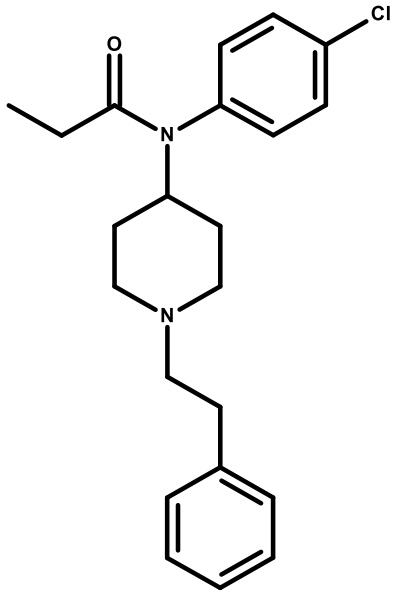
1. Only determines class of drug
2. False positives (ex. Sodium azide)
3. False negative (ex. nonreactive NPS)

Novel Psychoactive Substances

- NPS were created to mimic known illicit drugs
  - Legal highs and bath salts
- Due to change in structure from common drugs, screening for these drugs becomes complex
  - Limited information on NPS related to cross-reactivity on immunoassay
  - Often not included in screening scope using mass spectrometry methods



Fentanyl



Para-Chloro fentanyl

Previous Research

Journal of
Analytical Toxicology

Volume 40, Issue 2
March 2016

Article Contents

JOURNAL ARTICLE

ELISA Detection of Phenazepam, Etizolam, Pyrazolam, Flubromazepam, Diclazepam and Delorazepam in Blood Using Immunalysis® Benzodiazepine Kit

Lauren C. O'Connor ✉, Hazel J. Torrance, Denise A. McKeown

Journal of Analytical Toxicology, Volume 40, Issue 2, March 2016, Pages 159–161, https://doi-org.proxyiub.uits.iu.edu/10.1093/jat/bkv122
Published: 29 October 2015

Abstract

Project Goals

- Describe the cross reactivity of NPS opioids, stimulants, benzodiazepines, and hallucinogens on commercially available immunoassay kits for the purpose of toxicological screening.
- Testing ability of ELISA plates to cross react with NPS in authentic blood samples.

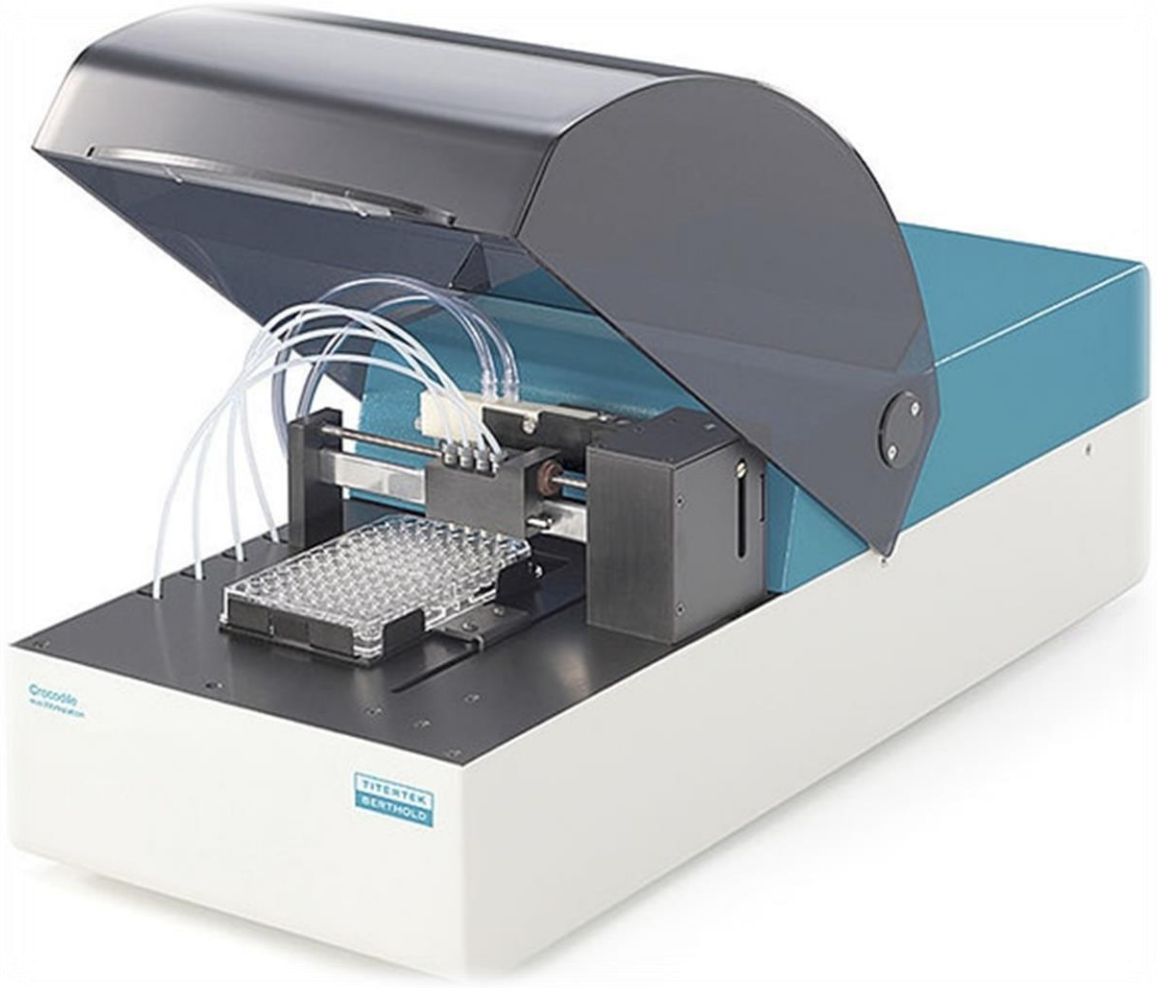
Testing Methods

Sample Preparation

- Blank blood samples were fortified with either an NPS or a reference drug
- The concentration ranges varied between drug class:
  - Novel opioids: 50-2000 ng/mL
  - Fentanyl analogs: 0.01-1 ng/mL
  - Novel stimulants: 20-2000 ng/mL
  - Novel benzodiazepines: 1-40 ng/mL
  - Novel hallucinogens: 10-1000 ng/mL
- Between 5 to 6 calibrators were prepared for each drug in the specified range

Enzyme Linked Immunosorbent Assay (ELISA)

- Five Neogen ELISA plates were used:
  - Opiate group - Morphine
  - Fentanyl
  - Amphetamine
  - Benzodiazepine group - Oxazepam
  - PCP
- Each plate was run with a matrix blank, reference drug and NPS
- All samples were run in duplicate
- All ELISA plates were run using Titertek-Berthold Crocodile Miniworkstation



Calculations

Optical densities recorded from ELISA were plotted against concentration for all NPS

Line of best fit was determined for each NPS graph

Determined the concertation where the NPS starts to cross react

Optical density of the reference drug at the cut off concertation inserted into the line of best fit equation.

Calculated concertation inserted into % cross reactivity equation

$$\%\ Cross\ Reactivity = \left(\frac{[Reference\ Drug]}{[NPS]}\right) * 100\%$$

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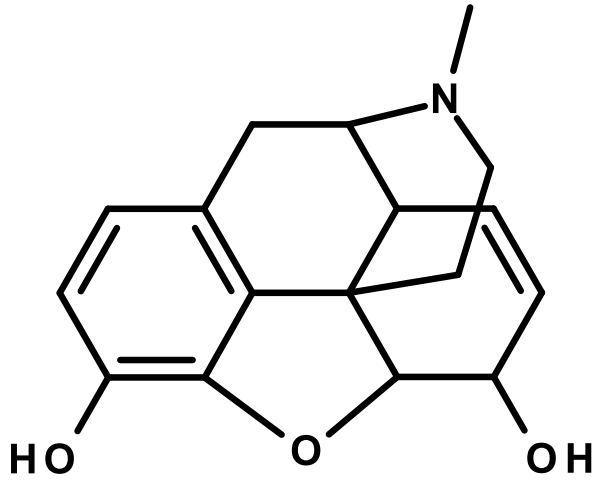
Results

Novel Opioids

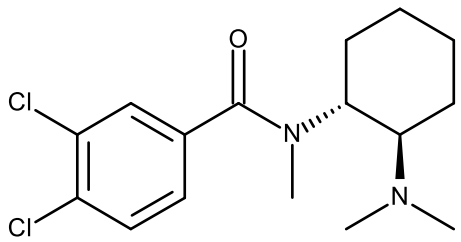
Reference Drug: Morphine

Cut-off concentration: 50 ng/mL

Conc. Range Tested: 50-2000 ng/mL



Novel Opioid Cross-Reactivity



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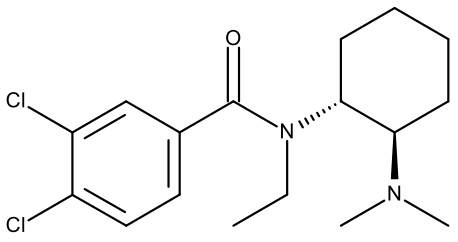

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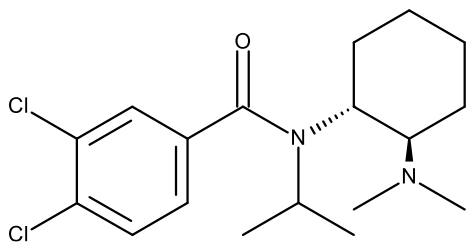

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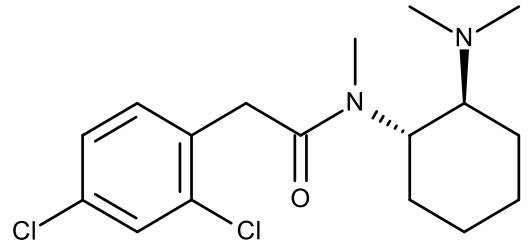

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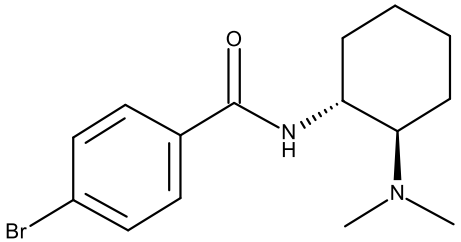

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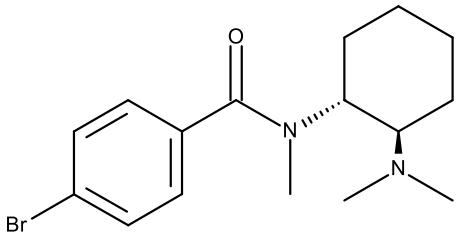

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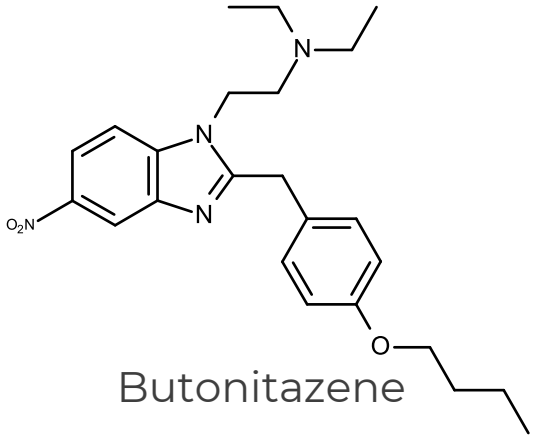
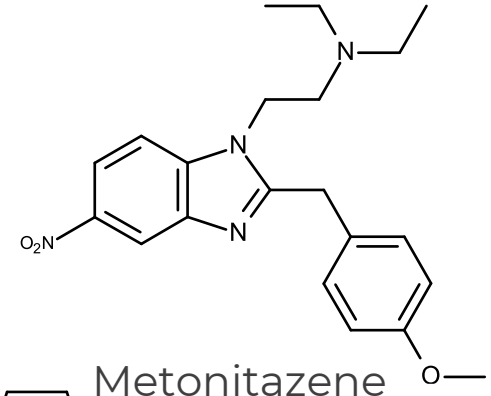
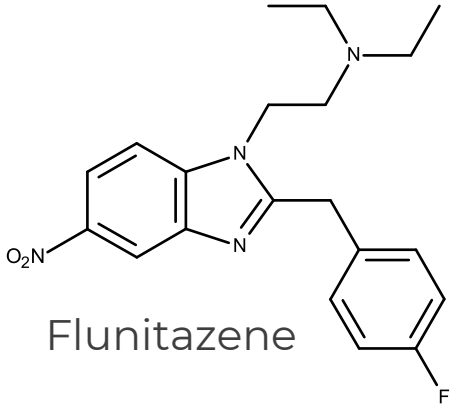


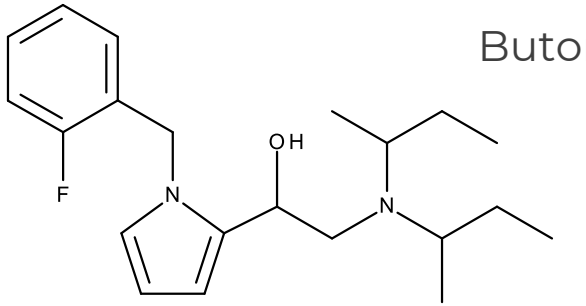
Butonitazene



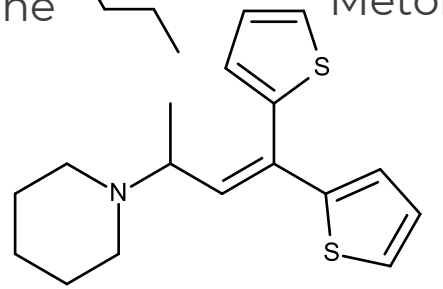
Metonitazene



Flunitazene

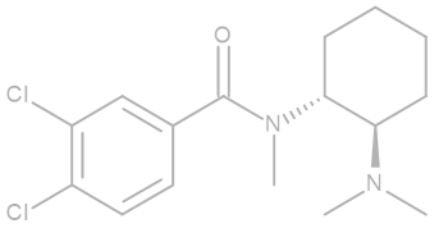


2-Fluoro viminol



Piperidylthiambutene

Novel Opioid Cross-Reactivity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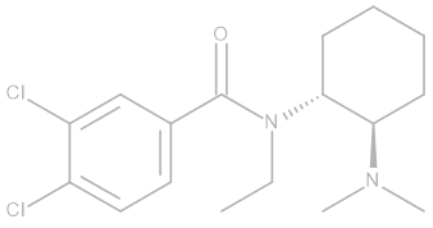
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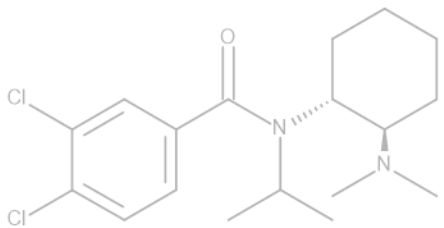
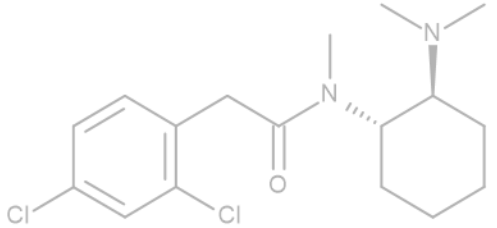

Isopropyl U-47700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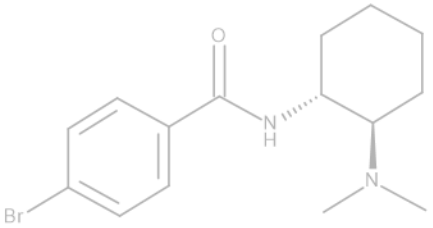
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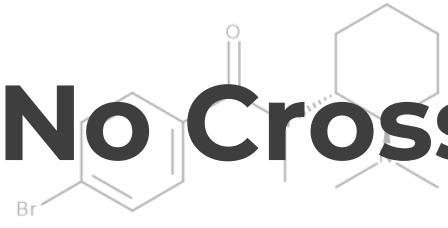
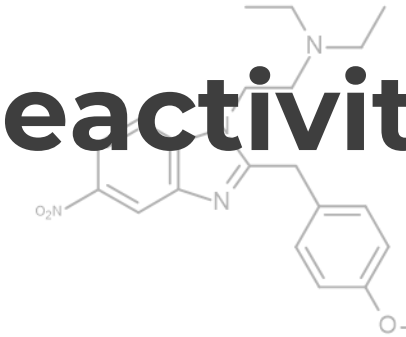
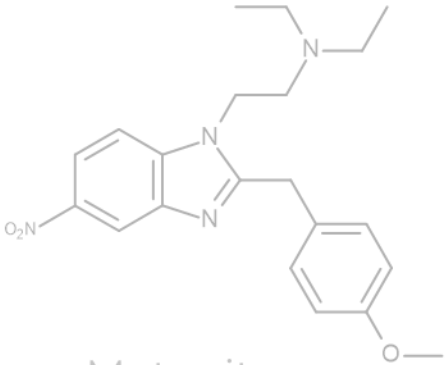
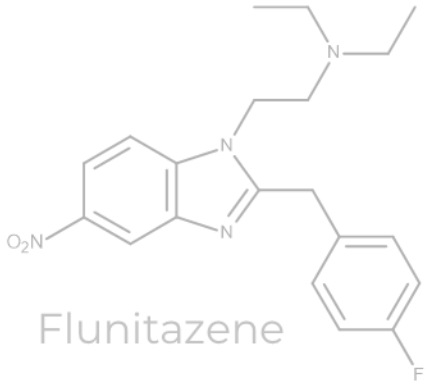
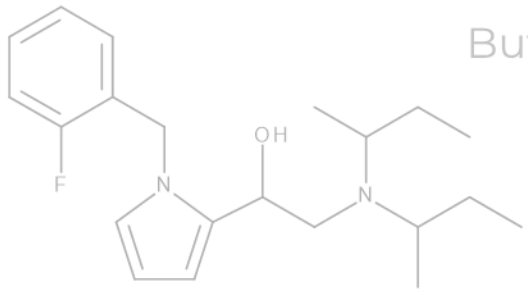

N-methyl U-47931E

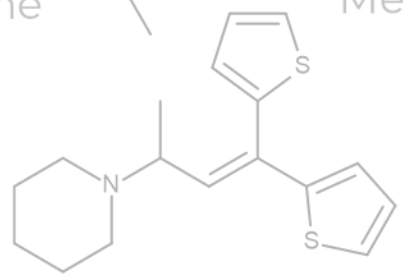
No Cross Reactivity


Butonitazene


Metonitazene


Flunitazene


2-Fluoro viminol

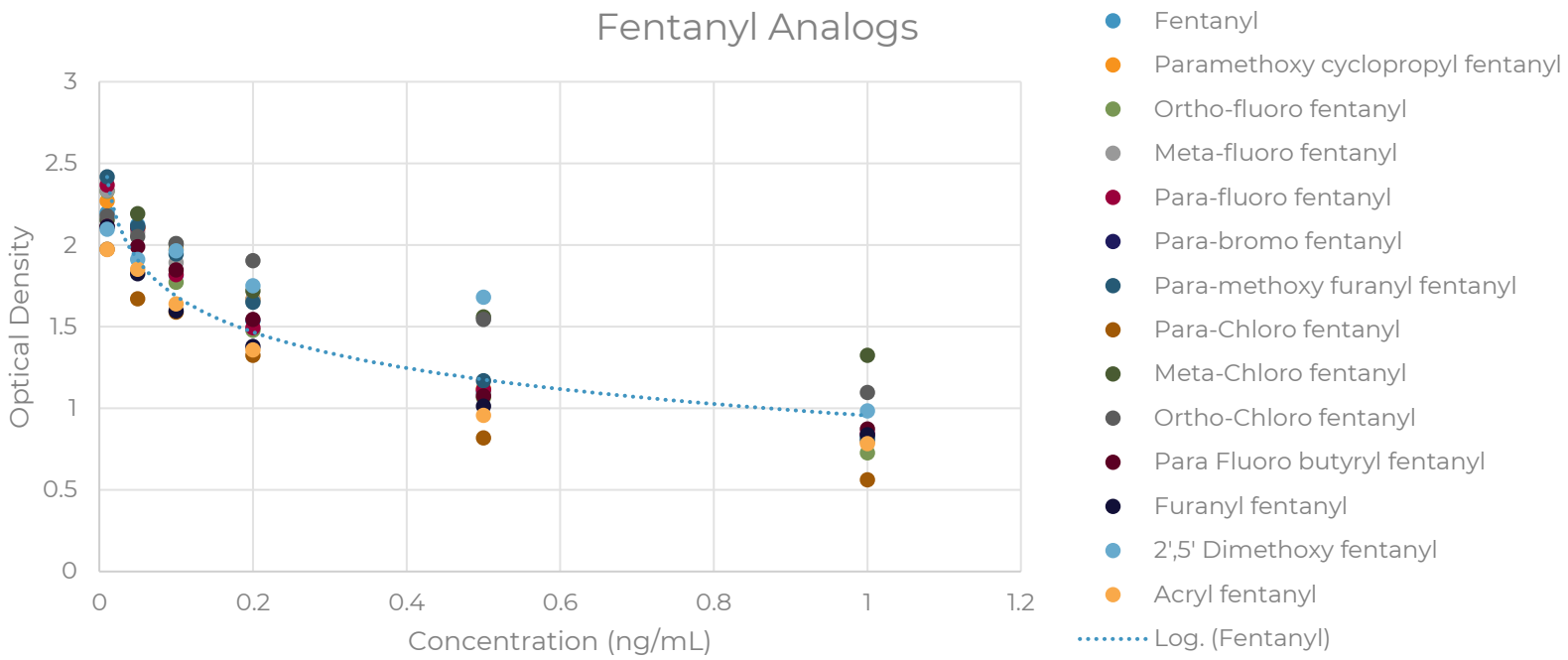

Piperidylthiambutene

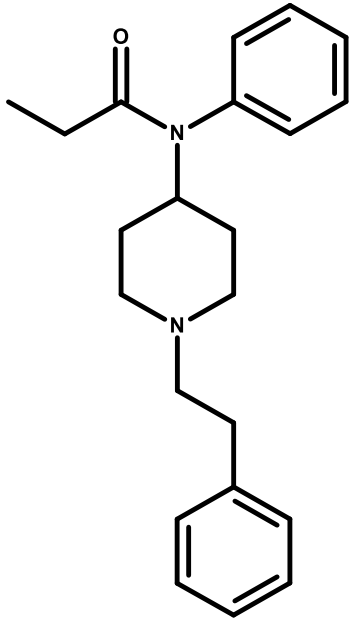
Fentanyl Analogs

Reference Drug: Fentanyl

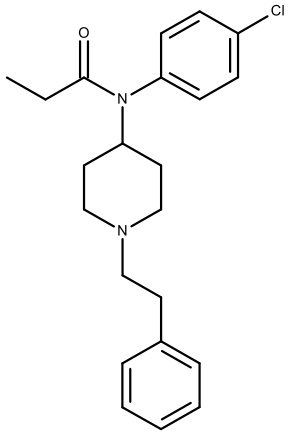
Cut-off Concentration: 0.5 ng/mL

Conc. Range Tested: 0.01-1 ng/mL

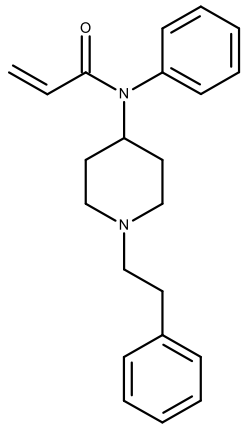




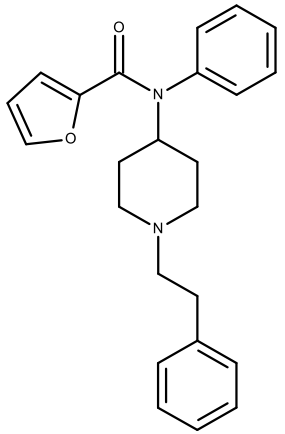
Fentanyl Analog Cross Reactivity



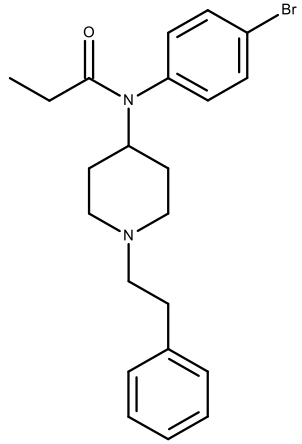
Para-chloro fentanyl



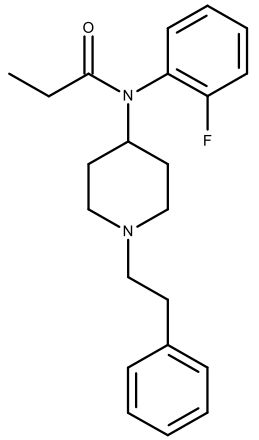
Acryl fentanyl



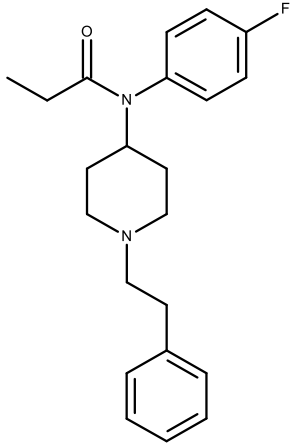
Furanyl fentanyl



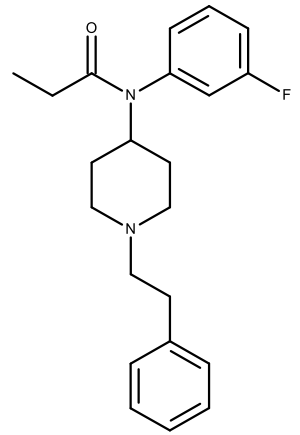
Para-bromo fentanyl



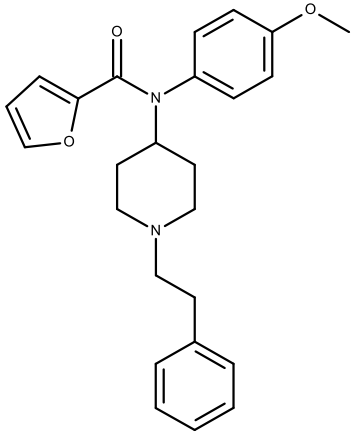
Ortho-fluoro fentanyl



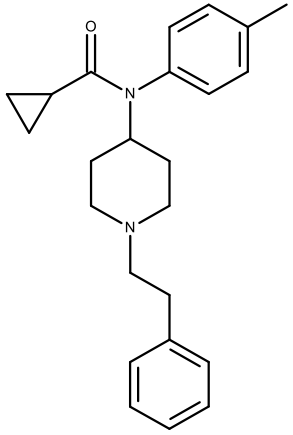
Para-fluoro fentanyl



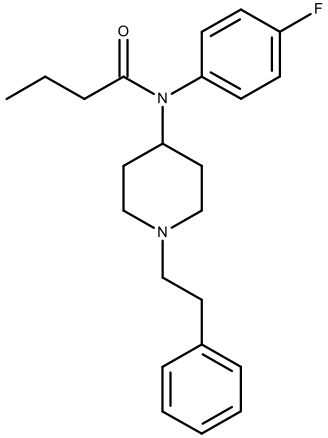
Meta-fluoro fentanyl



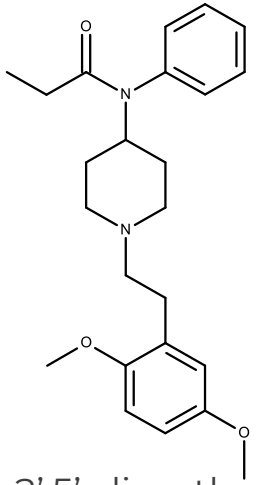
Para-methoxy furanyl fentanyl



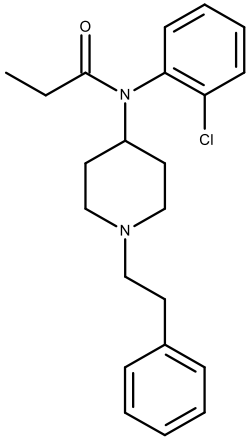
Para-methyl cyclopropyl fentanyl



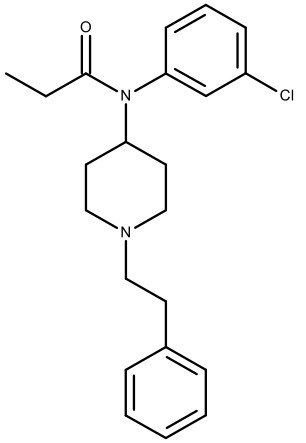
Para-fluoro butyryl fentanyl



2’,5’-dimethoxy fentanyl

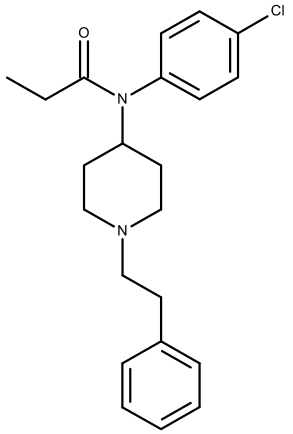


Ortho-chloro Fentanyl

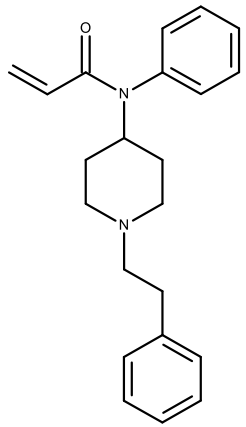


Meta-chloro fentanyl

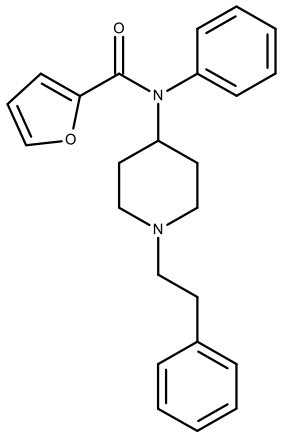
Fentanyl Analog Cross Reactivity



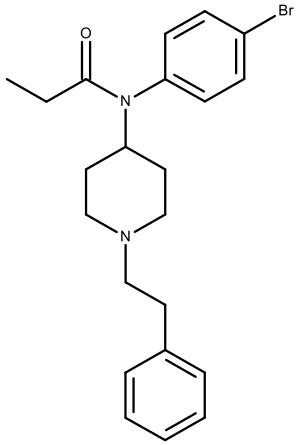
Para-chloro fentanyl
178%



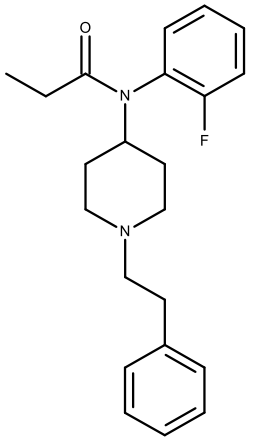
Acryl fentanyl
164%



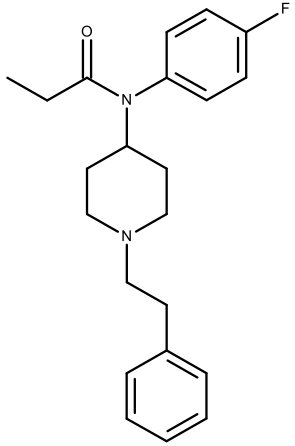
Furanyl fentanyl
103%



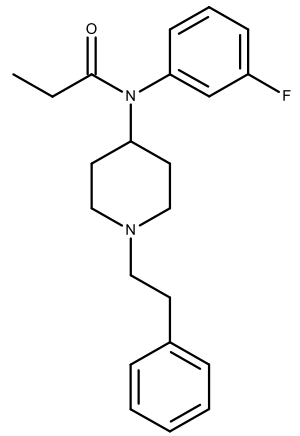
Para-bromo fentanyl
102%



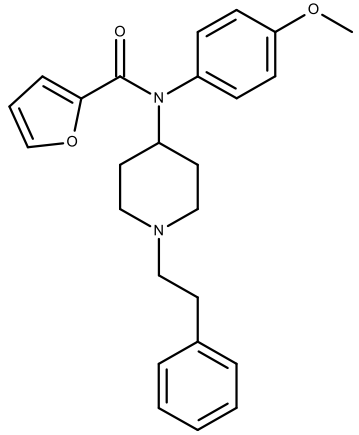
Ortho-fluoro fentanyl
101%



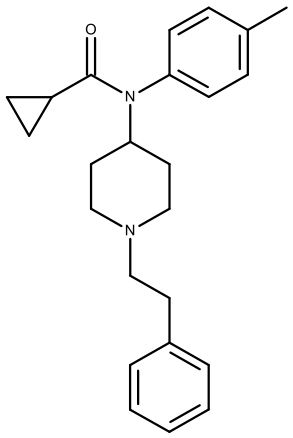
Para-fluoro fentanyl
84.1%



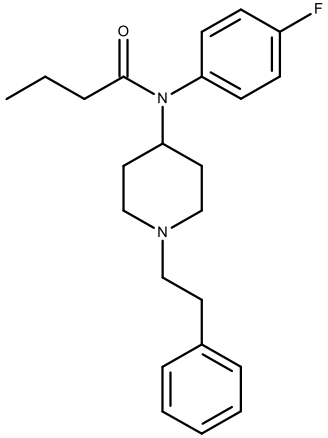
Meta-fluoro fentanyl
78.2%



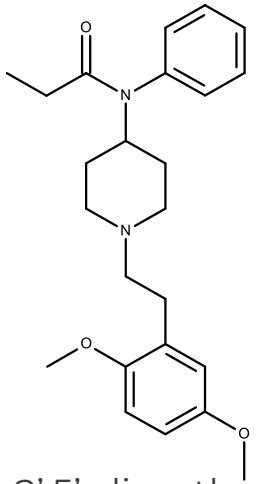
Para-methoxy furanyl fentanyl
72.8%



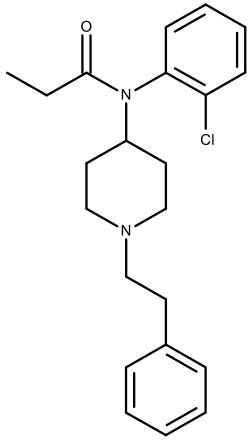
Para-methyl cyclopropyl fentanyl
71.8%



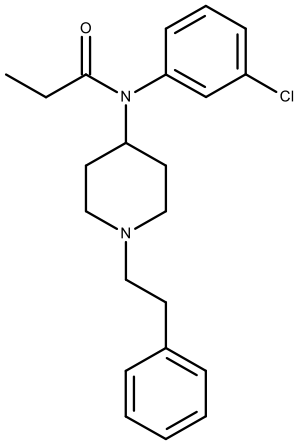
Para-fluoro butyryl fentanyl
70.1%



2',5'-dimethoxy fentanyl
15.2%



Ortho-chloro Fentanyl
14.7%



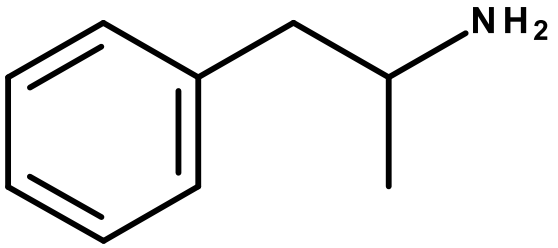
Meta-chloro fentanyl
8.31%

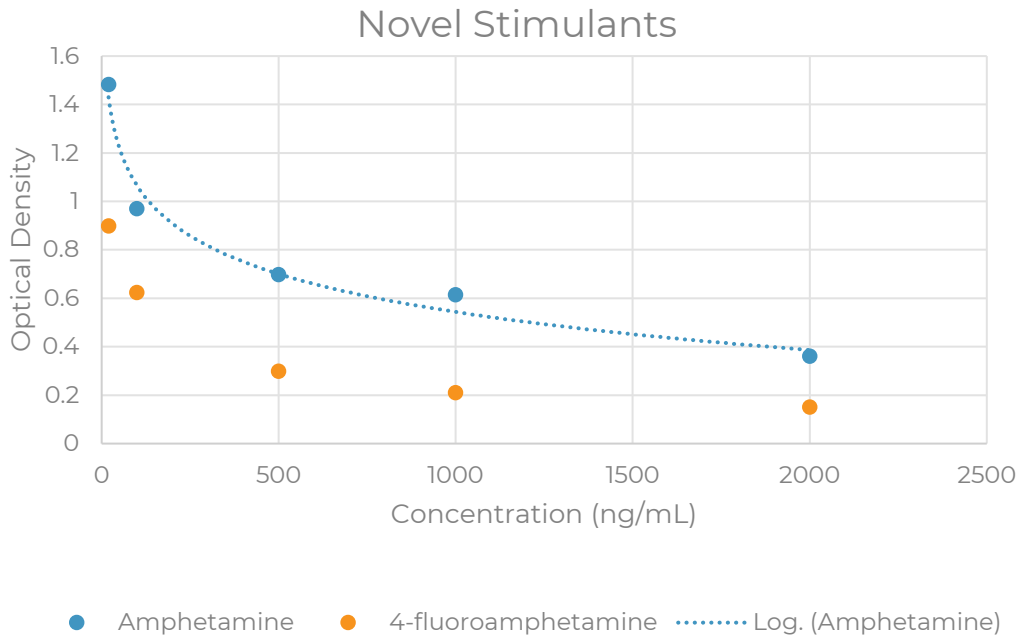
Novel Stimulants

Reference Drug: Amphetamine

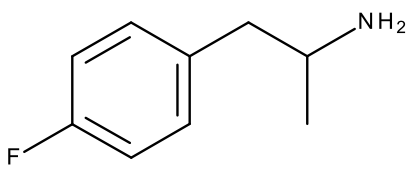
Cut-off Concentration: 20 ng/mL

Conc. Range Tested: 20-2000 ng/mL

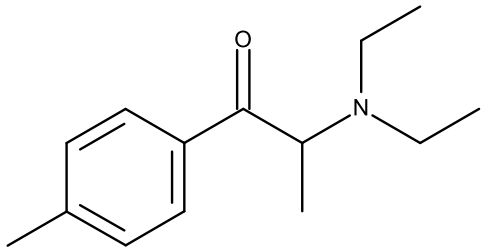




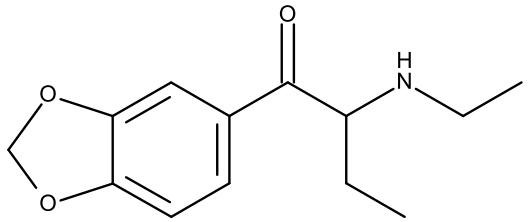
Novel Stimulants Cross Reactivity



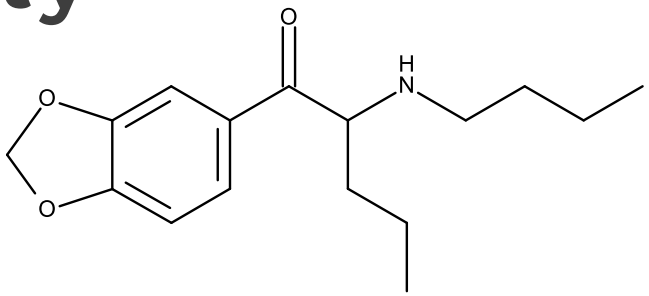
4-fluoroamphetamine



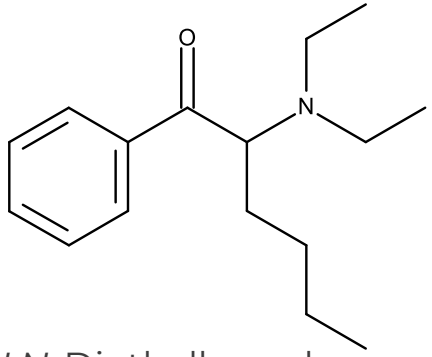
4-methyldiethcanthinone



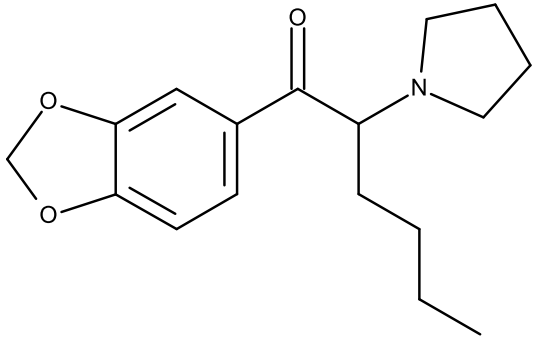
Eutylone



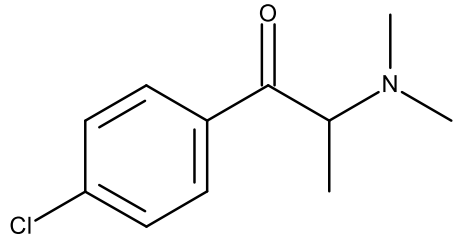
N-Butylpentylone



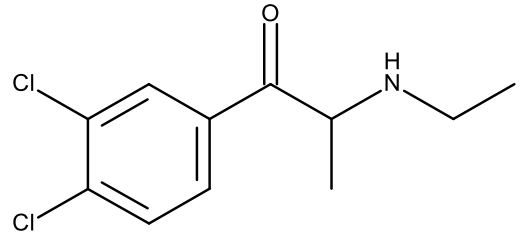
N,N-Diethylhexedrone



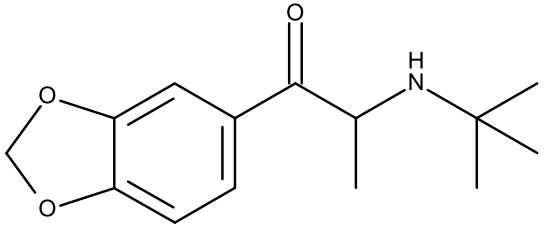
3,4-Methylenedioxy-α-pyrrolidinohexanophenone (MPDHP)



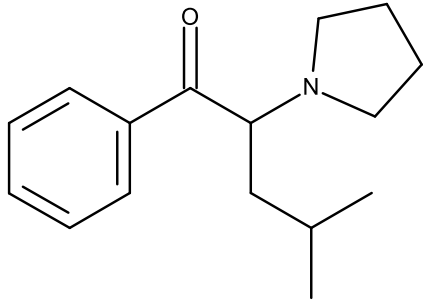
4-Chlorodimethylcathinone



3,4-Dichloroethcathinone

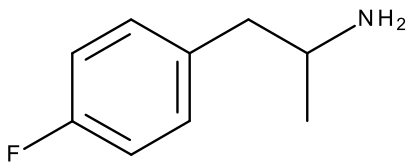


3’,4’-Methylenedioxy-N-tert-butylcathinone (Tertylone)

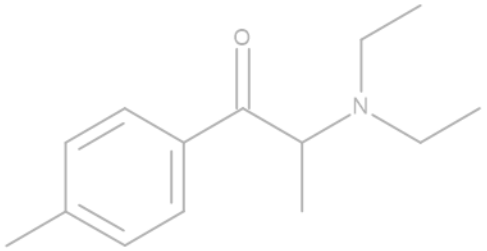


α-Pyrrolidinohexanophenone (α-PiHP)

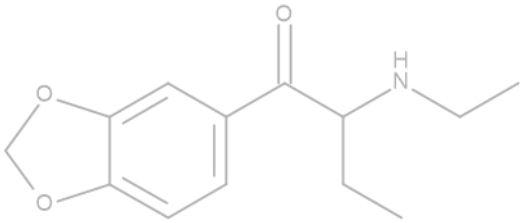
Novel Stimulants Cross Reactivity



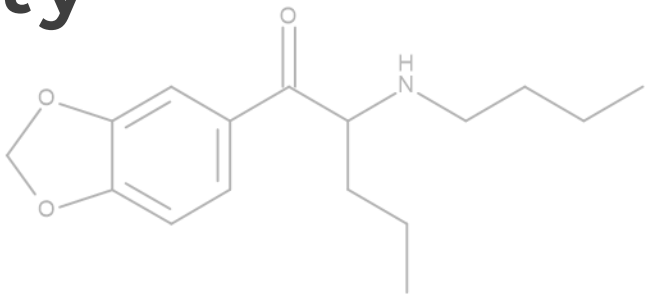
4-fluoroamphetamine
3,350%



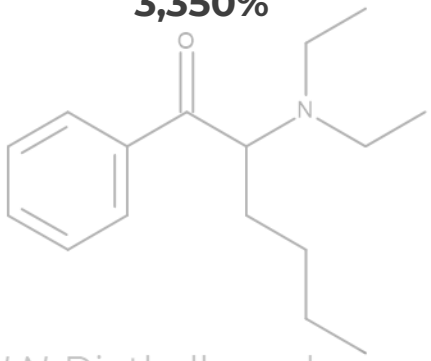
4-methyldiethcanthinone



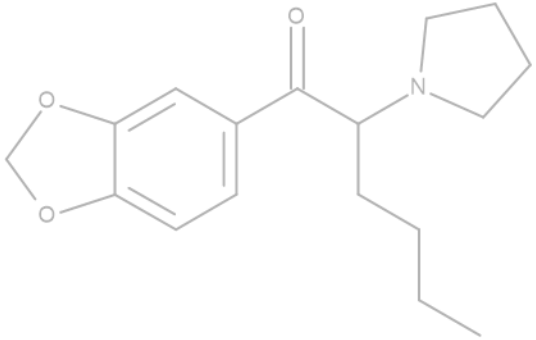
Eutylone



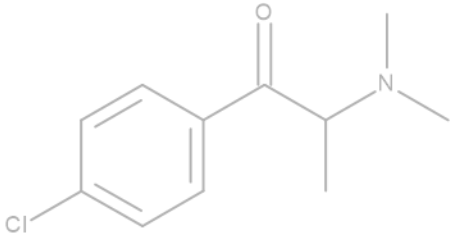
N-Butylpentylone



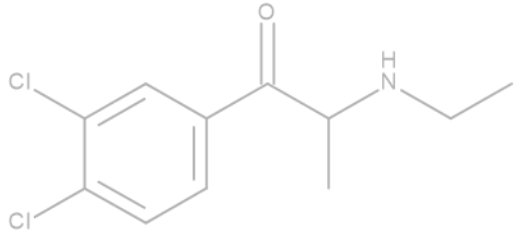
N,N-Diethylhexedrone



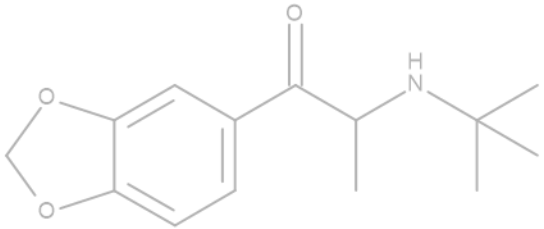
3,4-Methylenedioxy-α-pyrrolidinohexanophenone (MPDHP)



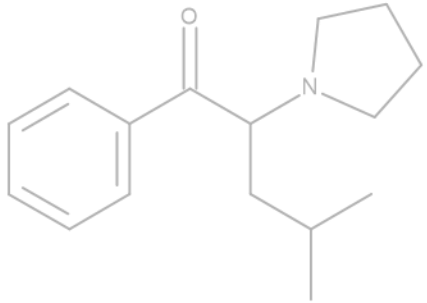
4-Chlorodimethylcathinone



3,4-Dichloroethcathinone



3’,4’-Methylenedioxy-N-tert-butylcathinone (Tertylone)



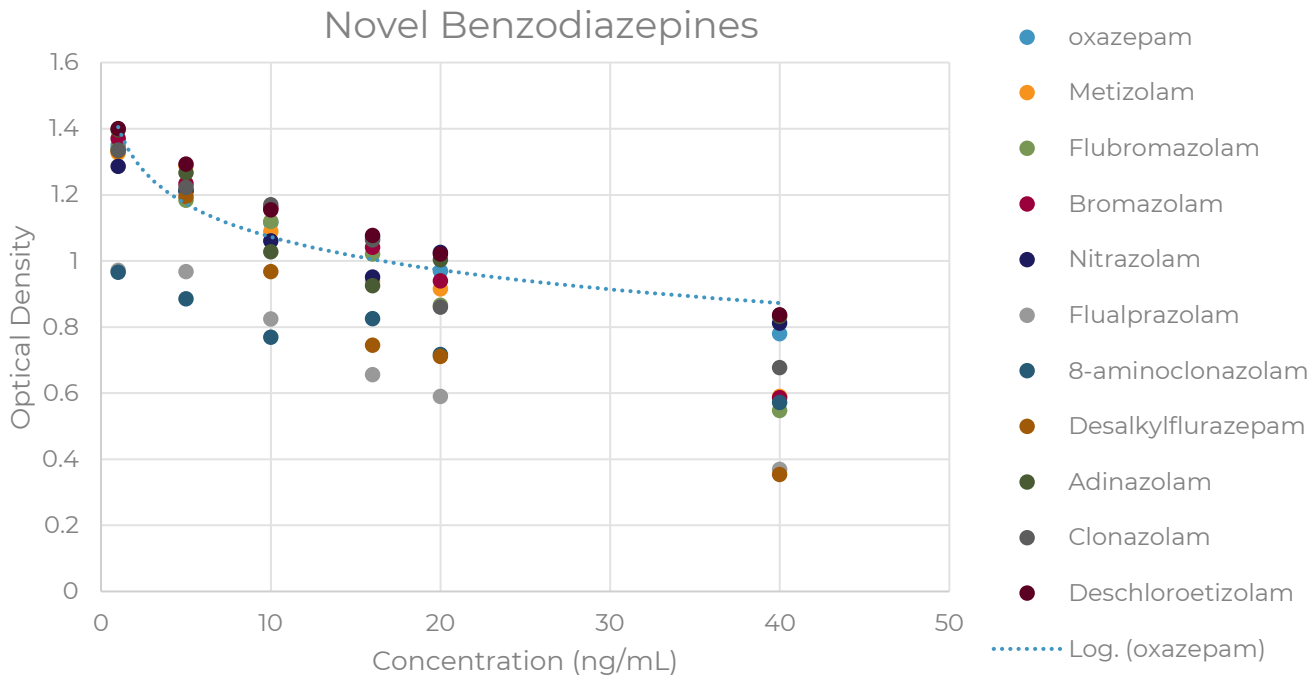
α-Pyrrolidinohexanophenone (α-PiHP)

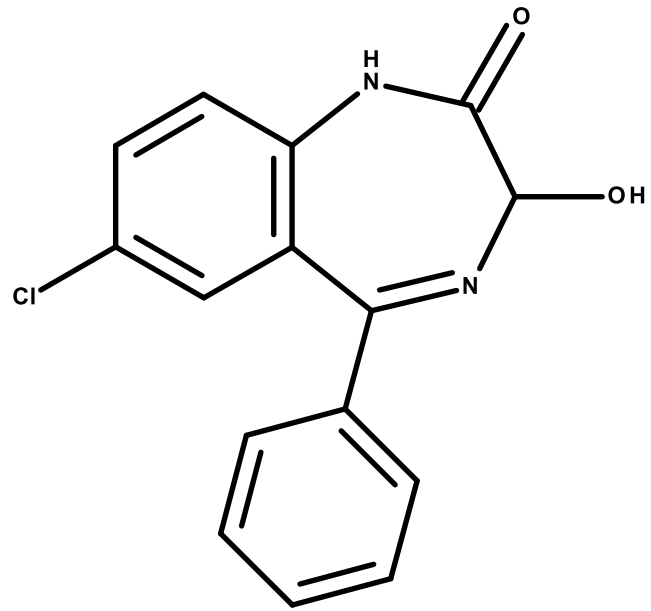
Novel Benzodiazepines

Reference Drug: Oxazepam

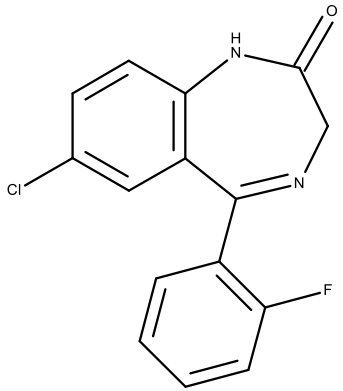
Cut-off Concentration: 40 ng/mL

Conc. Range Tested: 1-40 ng/mL

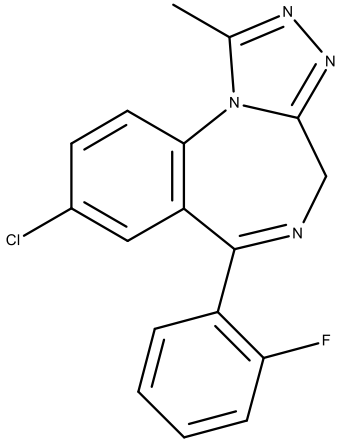




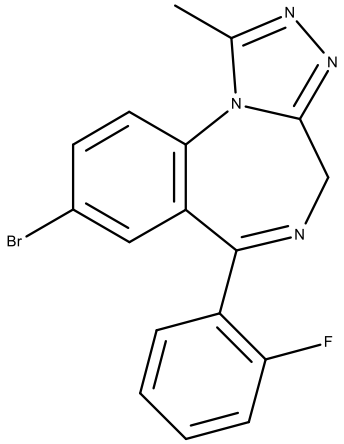
Novel Benzodiazepine Cross Reactivities



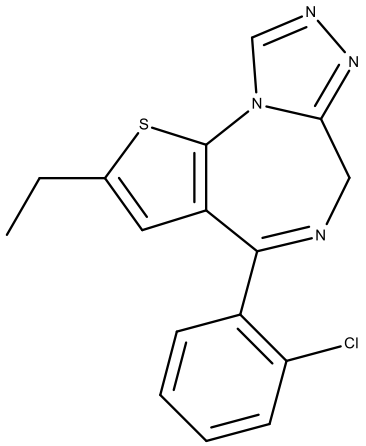
Desalkylflurazepam



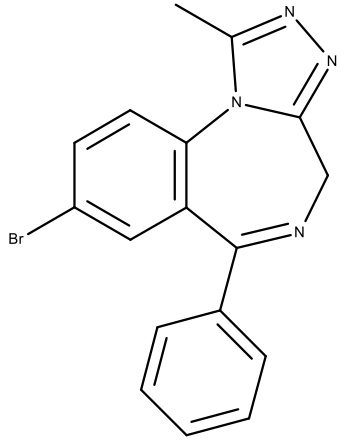
Flualprazolam



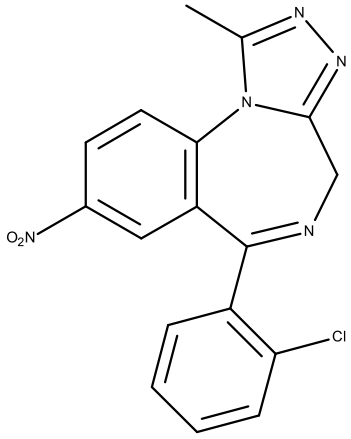
Flubromazolam



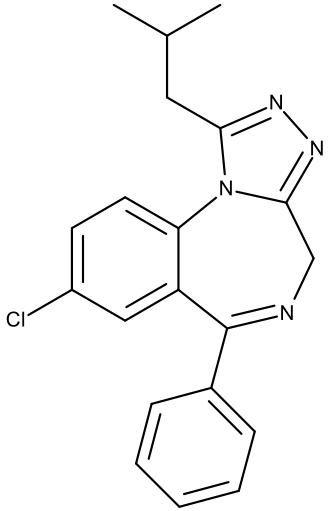
Metizolam



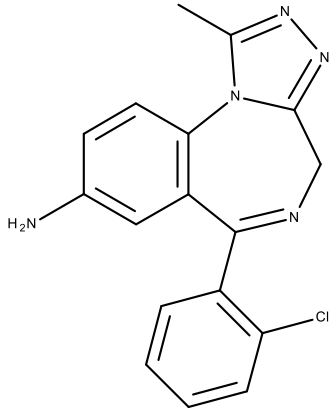
Bromazolam



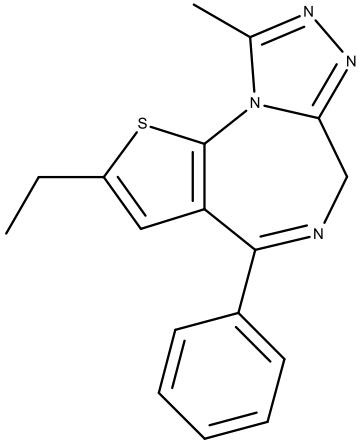
Clonazolam



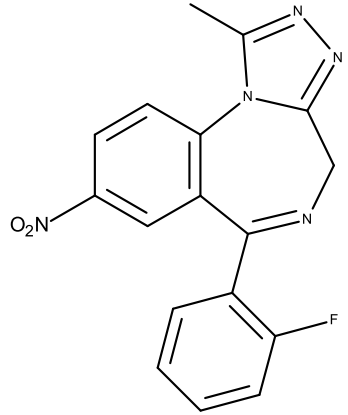
Adinazolam



8-aminoclonazolam

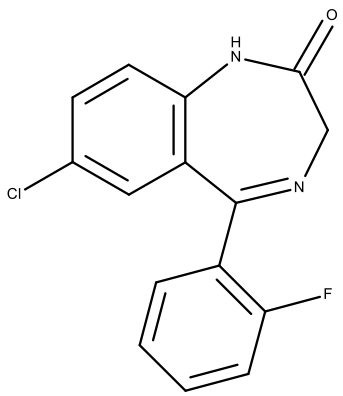


Deschloroetizolam

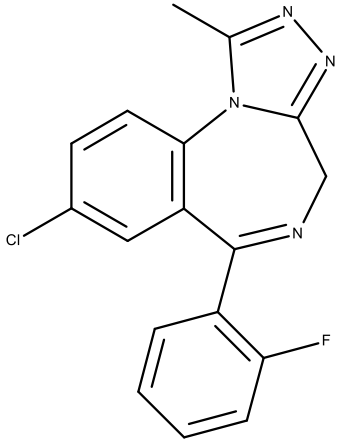


Nitrazolam

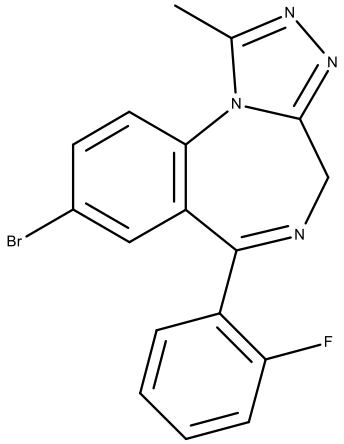
Novel Benzodiazepine Cross Reactivities



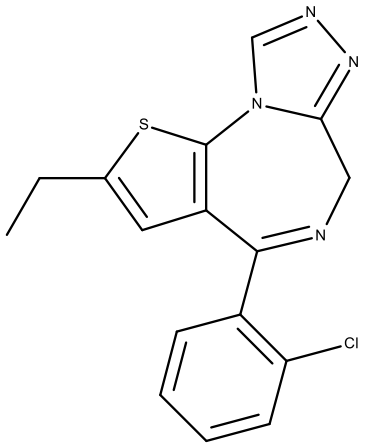
Desalkylflurazepam
263%



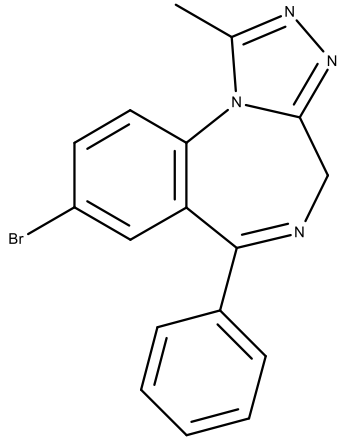
Flualprazolam
173%



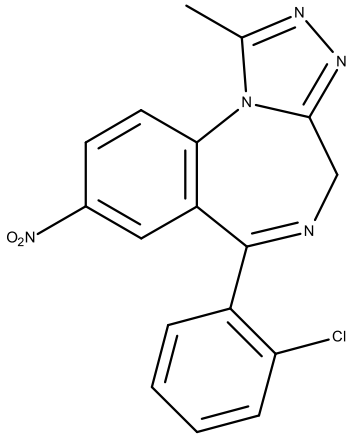
Flubromazolam
127%



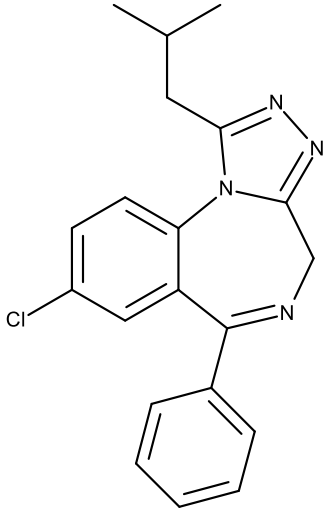
Metizolam
120%



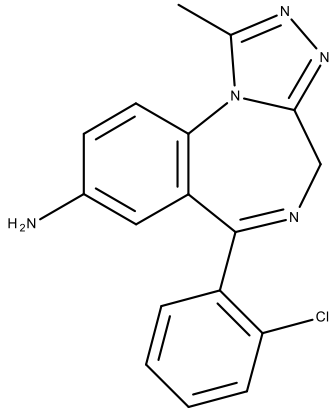
Bromazolam
99.8%



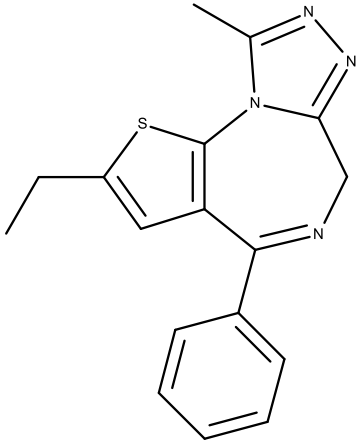
Clonazolam
87.3%



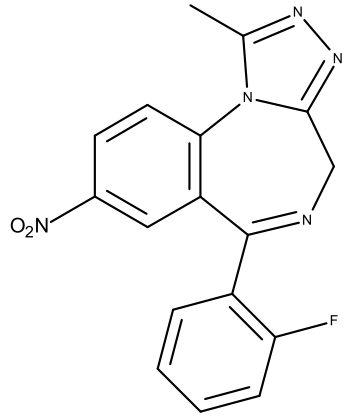
Adinazolam
69.6%



8-aminoclonazolam
51.3%



Deschloroetizolam
41.6%



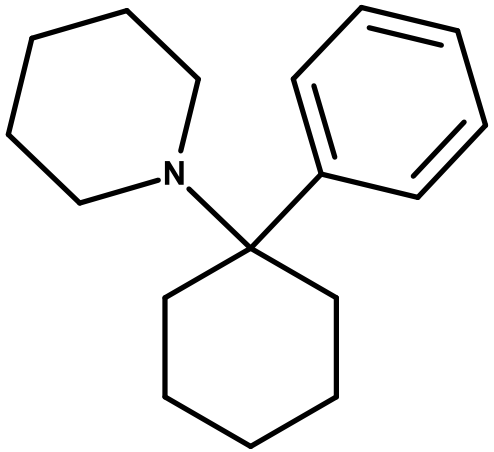
Nitrazolam
36.1%

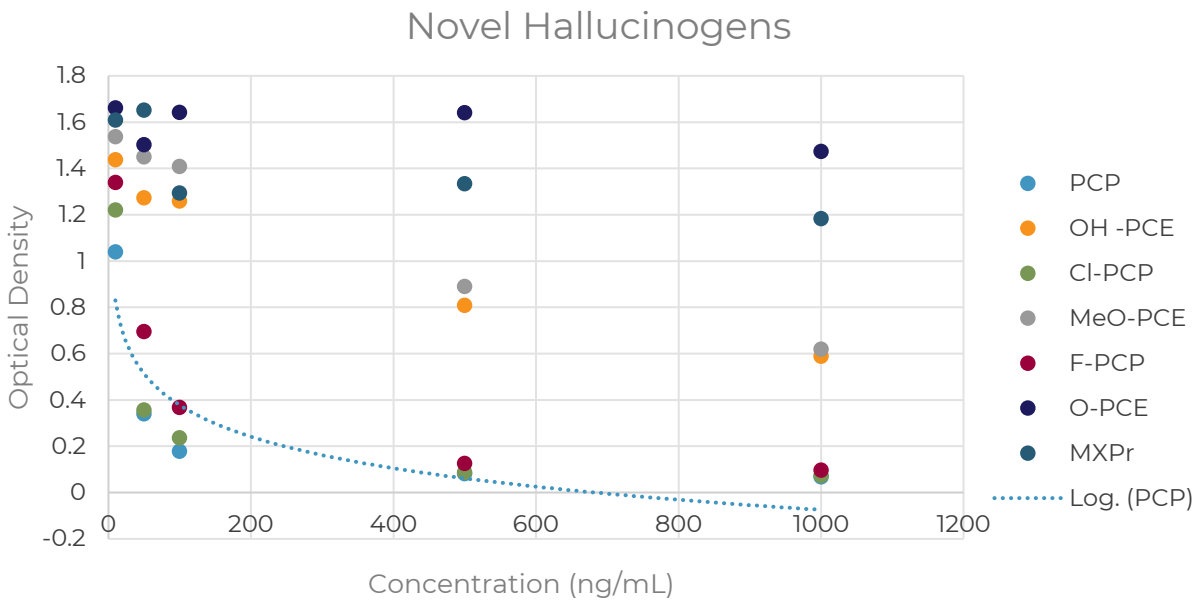
Novel Hallucinogens

Reference Drug: Phencyclidine (PCP)

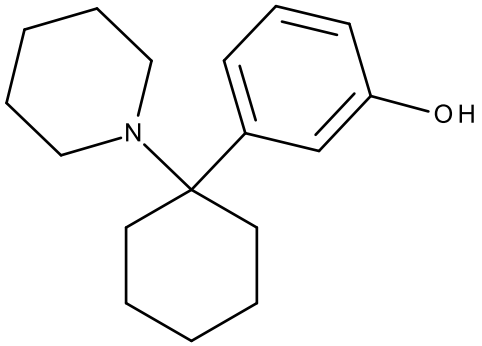
Cut-off Concentration: 10 ng/mL

Conc. Range Tested: 10-1000 ng/mL

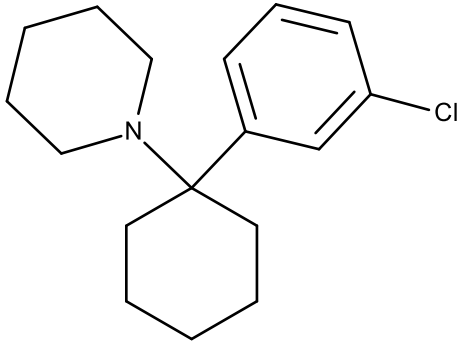




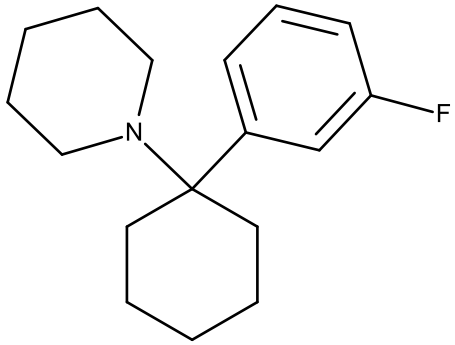
Novel Hallucinogens Cross Reactivity



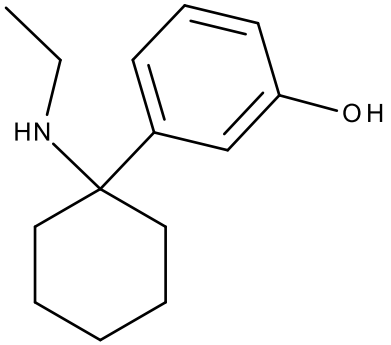
Hydroxy-PCP



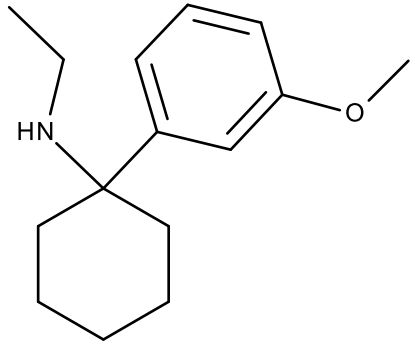
Chloro-PCP



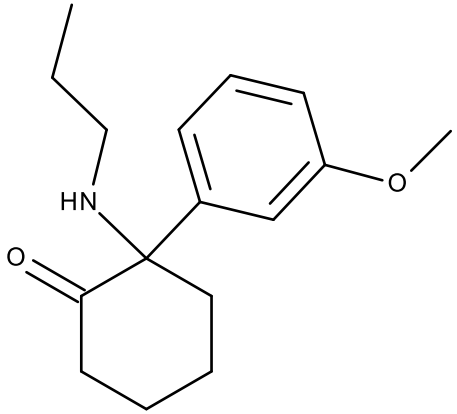
Fluoro-PCP



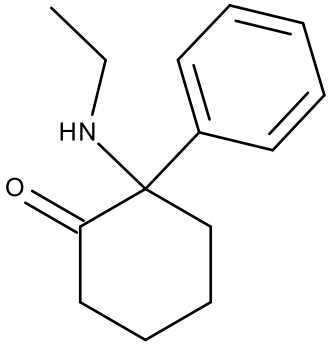
Hydroxy-PCE



Methoxy-PCE

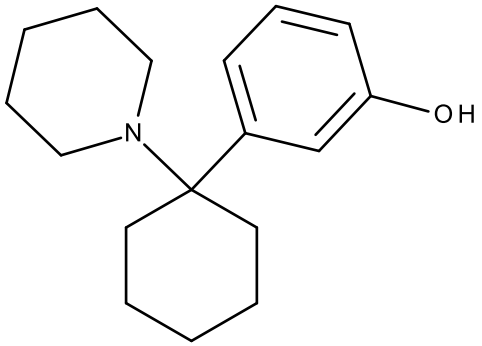


Methoxpropamine

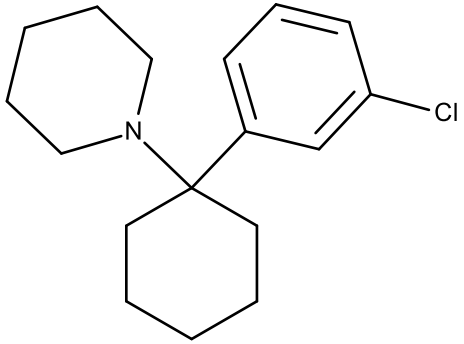


Deschloro-*N*-ethyl ketamine

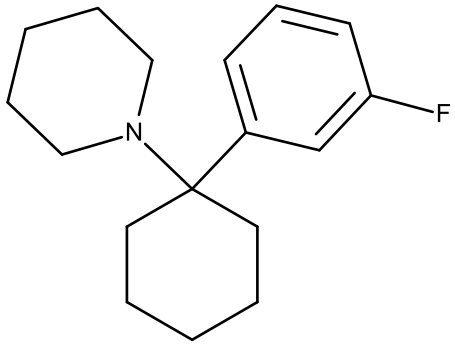
Novel Hallucinogens Cross Reactivity



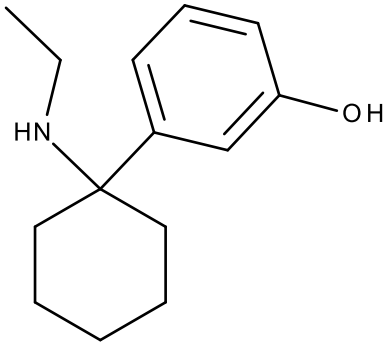
Hydroxy-PCP
151.7%



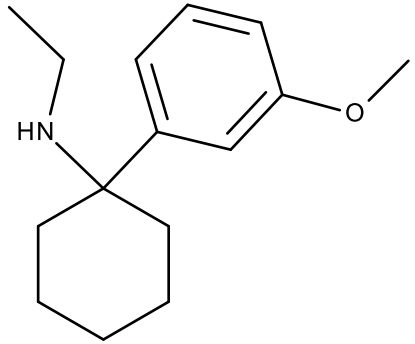
Chloro-PCP
136.6%



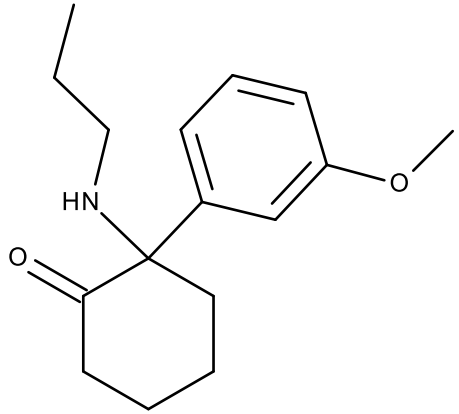
Fluoro-PCP
56.6%



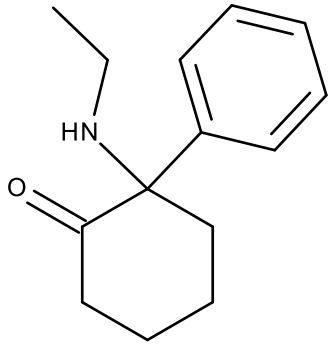
Hydroxy-PCE
6.85%



Methoxy-PCE
4.18%



Methoxpropamine
No Cross Reactivity



Deschloro-*N*-ethyl ketamine
No Cross Reactivity

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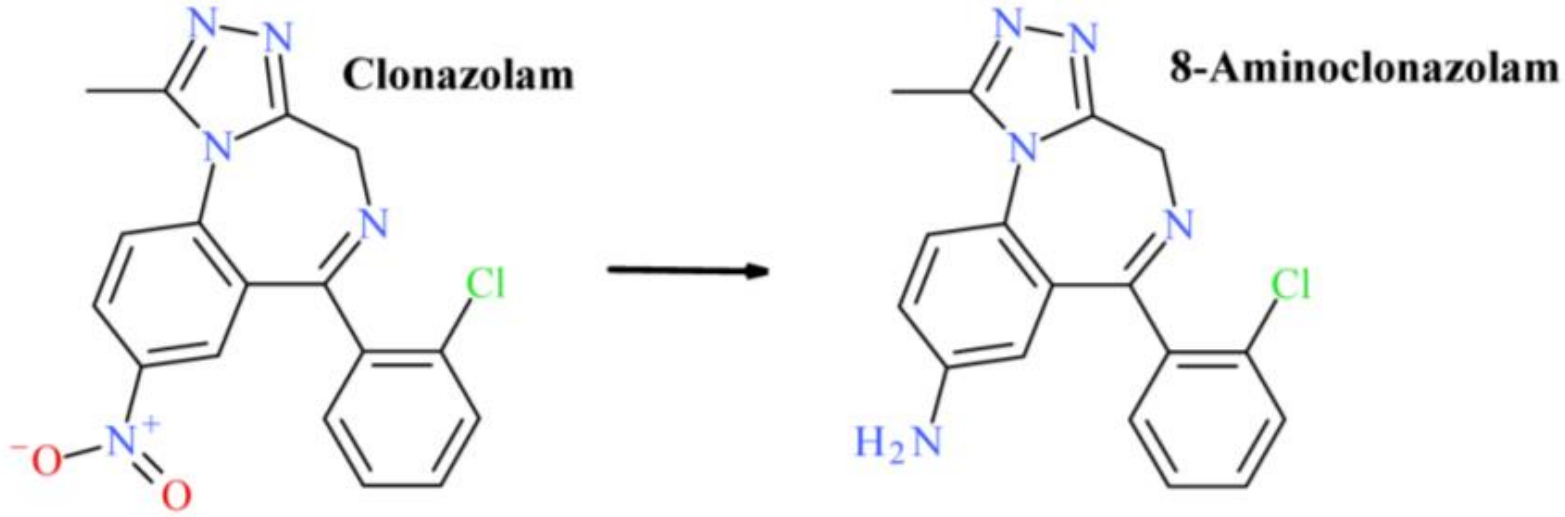
Implementation

Authentic Samples

- Discarded and deidentified blood samples were screened for the presence of NPS using LC-QTOF
  - January 2020 to December 2021
- 28 samples total contained an NPS within the scope of this research
  - 19 contained novel benzodiazepines
    - 10 – clonazolam/8-aminoclonazolam, 5 – flualprazolam, 4 – flubromazolam
  - 9 contained novel stimulants
    - Eutylone
- Samples were run on ELISA plates with reference drug calibrators and matrix blank

Screening Results for Authentic Samples

NPS	Number of Cases	Number of Positives	Detection Rate
Clonazolam/ 8-Aminoclonazolam	10	4	40%
Flubromazolam	4	2	50%
Flualprazolam	5	0	0%
Eutylone	9	0	0%



Quantitative Data

Drug	Number of Samples	Number of Screen Positives	Conc. Range (ng/mL)	Calculated Conc. of Cross Reactivity (ng/mL)
Clonazolam/8-aminoclonazolam	10	0	<5.0-9.3 (n=3)	45.8
Flubromazolam	4	2	11, 56 (n=2)	31.4
Flualprazolam	5	0	11 (n=1)	23.1
Eutylone	9	0	300, 940 (n=2)	No CR

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Discussion and Conclusions

Discussion

- Total of 53 NPS tested across five classes
  - 29 drugs had cross reactivity
    - 13 fentanyl analogs
    - 10 novel benzodiazepines
    - 5 novel hallucinogens
    - 1 novel stimulant
    - 0 novel opioids
- The percentages of cross reactivity for drugs that did cross react ranged from 4.18% to 3,350%
- The remaining 24 NPS showed no cross reactivity to the plate
  - Most were novel opioids and novel stimulants

Conclusion

- Structural similarity to the reference drug good indicator for cross reactivity
  - –Fentanyl analogs and novel benzodiazepines
- False negative results are likely due to large number of drugs with no cross reactivity to ELISA plates.
- Recommend using alternative screening methods if NPS are suspected.
- If using ELISA, in house evaluation of cross reactivity should be conducted

Acknowledgements

- Carolina Noble
- NMS Labs
- CFSRE Staff

Questions?

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