

# Pharmacology and Toxicology of *N*-Pyrrolidino Etonitazene – A New Nitazene Synthetic Opioid Increasingly Observed in Forensic Cases

Sara E. Walton<sup>1\*</sup>, Marthe M. Vandeputte<sup>2</sup>, Alex J. Krotulski<sup>1</sup>, Donna Walther<sup>3</sup>, Grant C. Glatfelter<sup>3</sup>, Donna M. Papsun<sup>4</sup>, Barry K. Logan<sup>1,4</sup>, Michael H. Baumann<sup>3</sup>, Christophe P. Stove<sup>2</sup>

<sup>1</sup>Center for Forensic Science Research and Education, Willow Grove, PA

<sup>2</sup>Laboratory of Toxicology at Ghent University, Belgium

<sup>3</sup>Designer Drug Research Unit, National Institute on Drug Abuse, Baltimore, MD

<sup>4</sup>NMS Labs, Horsham, PA



## Disclosures

- I have no conflicts of interest to disclose.
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# Background





# Objective

Pair *in vitro* and *in vivo* methodologies to characterize the new synthetic opioid, *N*-pyrrolidino etonitazene

- Radioligand Binding Assays (*in vitro*)
- Activation Potential Assays (*in vitro*)
- Pharmacodynamic Studies (*in vivo*)
- Toxicology Method Development & Validation
- Evaluation of Medicolegal Death Investigation Cases

## Published Work


Archives of Toxicology

<https://doi.org/10.1007/s00204-022-03276-4>

ORGAN TOXICITY AND MECHANISMS



# Pharmacological evaluation and forensic case series of *N*-pyrrolidino etonitazene (etonitazepyne), a newly emerging 2-benzylbenzimidazole 'nitazene' synthetic opioid

Marthe M. Vandeputte<sup>1</sup> · Alex J. Krotulski<sup>2</sup> · Donna Walther<sup>3</sup> · Grant C. Glatfelter<sup>3</sup> · Donna Papsun<sup>4</sup> · Sara E. Walton<sup>2</sup> · Barry K. Logan<sup>2,4</sup> · Michael H. Baumann<sup>3</sup> · Christophe P. Stove<sup>1</sup> 

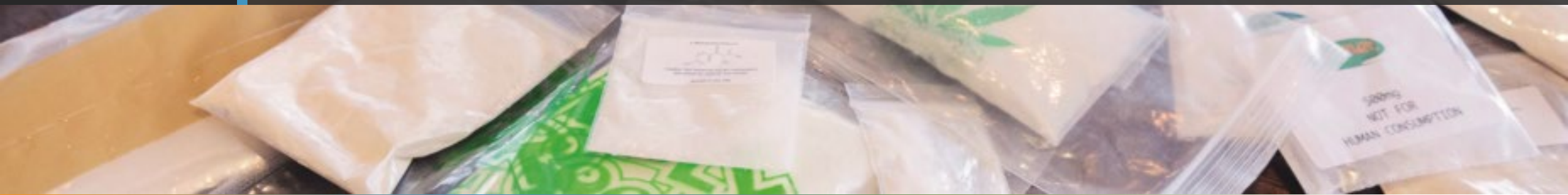
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# Pharmacodynamic Studies

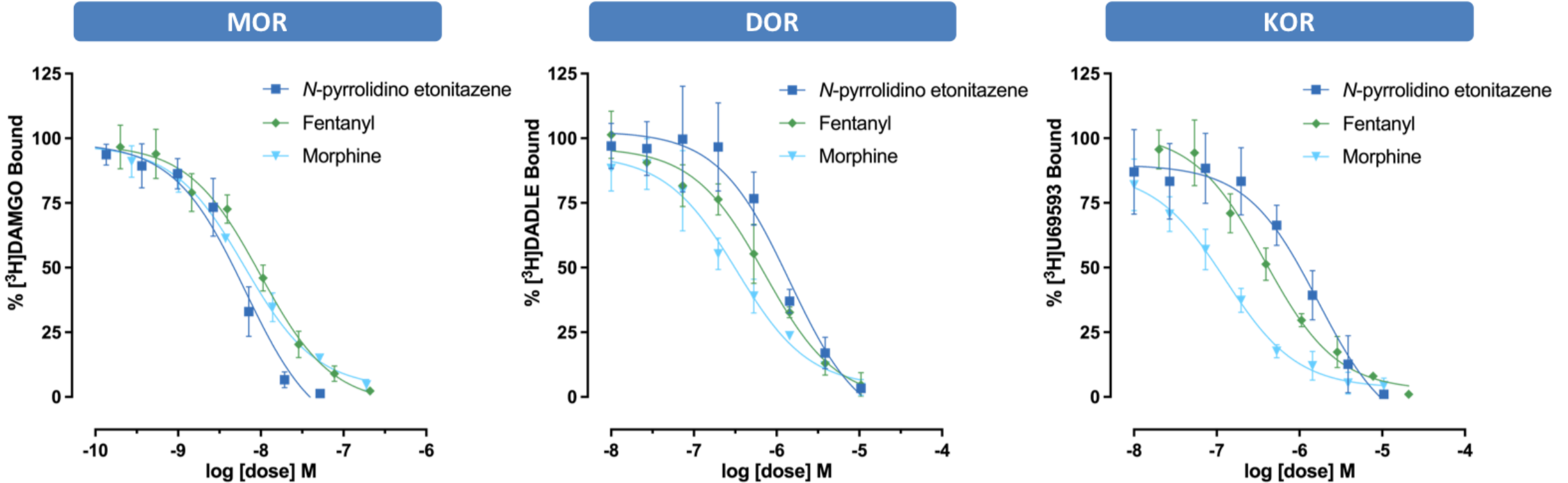


## Radioligand Binding – Rat Brain Tissue

- Opioid receptor binding assays
  - To determine affinity for MOR, DOR, KOR
- Radioligands (1 nM final concentration):
  - [<sup>3</sup>H]DAMGO:  $\mu$ -opioid receptor label
  - [<sup>3</sup>H]DADLE:  $\delta$ -opioid receptor label
  - [<sup>3</sup>H]U69,593:  $\kappa$ -opioid receptor label
- Non-specific binding determined by presence of 10  $\mu$ M naloxone
- Stock solution *N*-pyrrolidino etonitazene: 10 mM
  - Compared with fentanyl and morphine
- Radioactivity counted using Perkin Elmer MicroBeta2 liquid scintillation counter
  - $K_i$  values determined using non-linear regression analysis



# Opioid Receptor Affinity



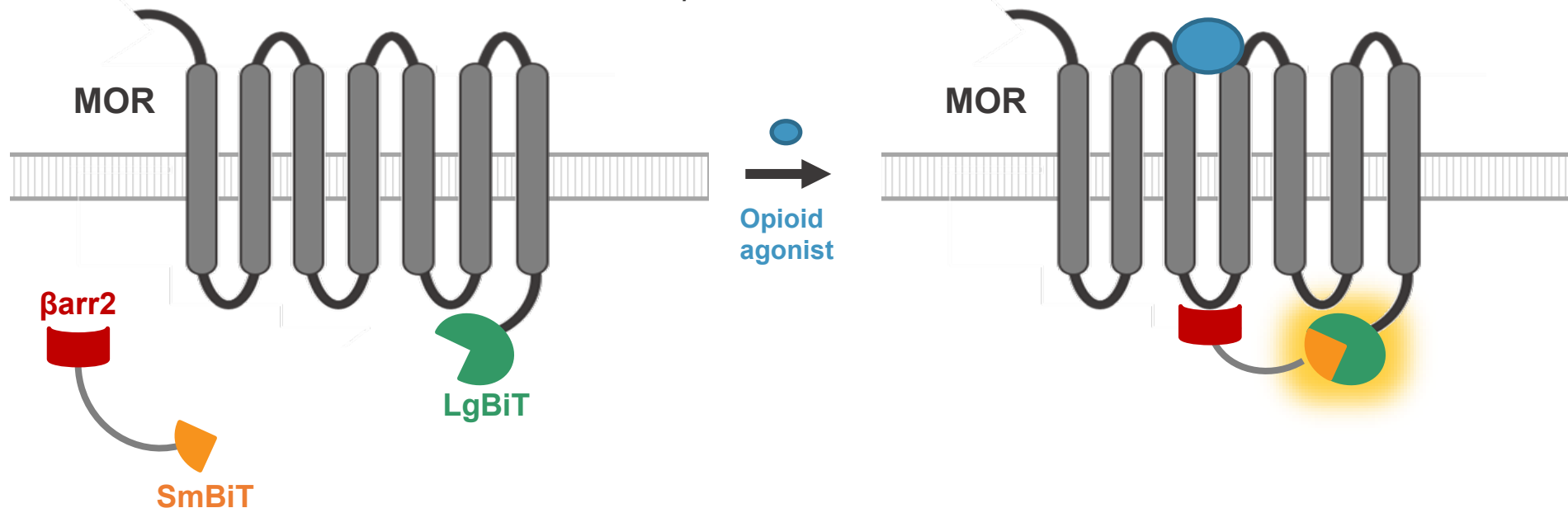
## Opioid Receptor Affinity

- MOR receptor affinity of *N*-pyrrolidino etonitazene comparable to morphine and greater than fentanyl
  - KOR and DOR affinity lower than morphine and fentanyl
  - Significant MOR selectivity over KOR and DOR

| Ki (nM)                              | MOR<br>[ <sup>3</sup> H]DAMGO | DOR<br>[ <sup>3</sup> H]DADLE | KOR<br>[ <sup>3</sup> H]U69,593 |
|--------------------------------------|-------------------------------|-------------------------------|---------------------------------|
| <i>N</i> -Pyrrolidino<br>Etonitazene | 4.09 ± 0.63                   | 959 ± 193                     | 980 ± 213                       |
| Fentanyl                             | 6.17 ± 0.82                   | 479 ± 76                      | 224 ± 33                        |
| Morphine                             | 3.99 ± 0.40                   | 220 ± 41                      | 74.4 ± 11.8                     |

## MOR Activation Potential Assays

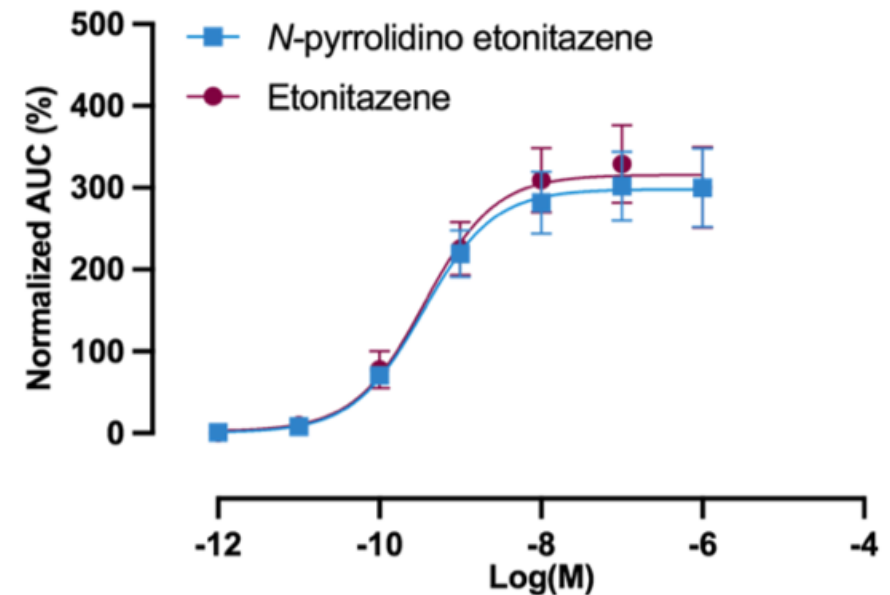
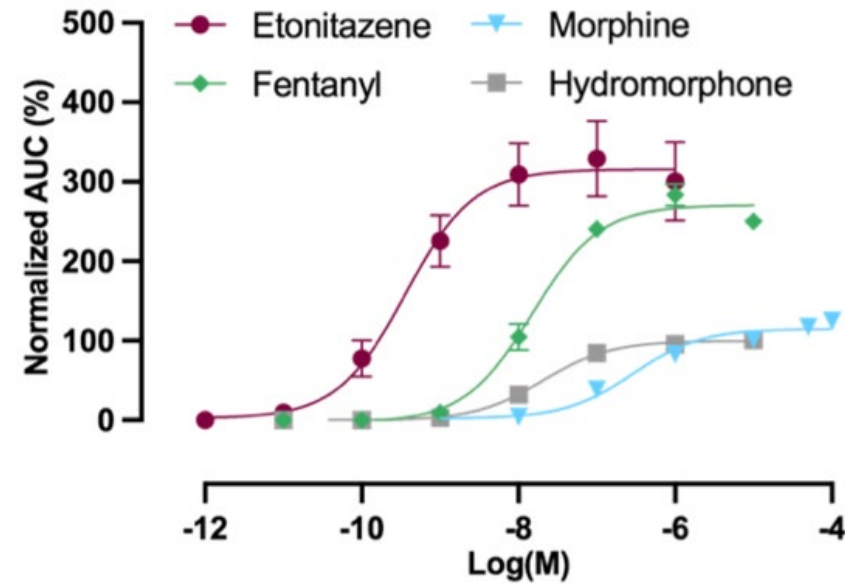
- NanoBiT® MOR- $\beta$ -Arrestin 2 Recruitment Assay
- Comparison to morphine, fentanyl, and etonitazene
  - Hydromorphone: reference drug
  - Drugs tested between 1 pM and 100  $\mu$ M
- Based on functional complementation of split nanoluciferase enzyme
  - Activation of MOR leads to recruitment of  $\beta$ -arrestin 2





## MOR Activation Potential

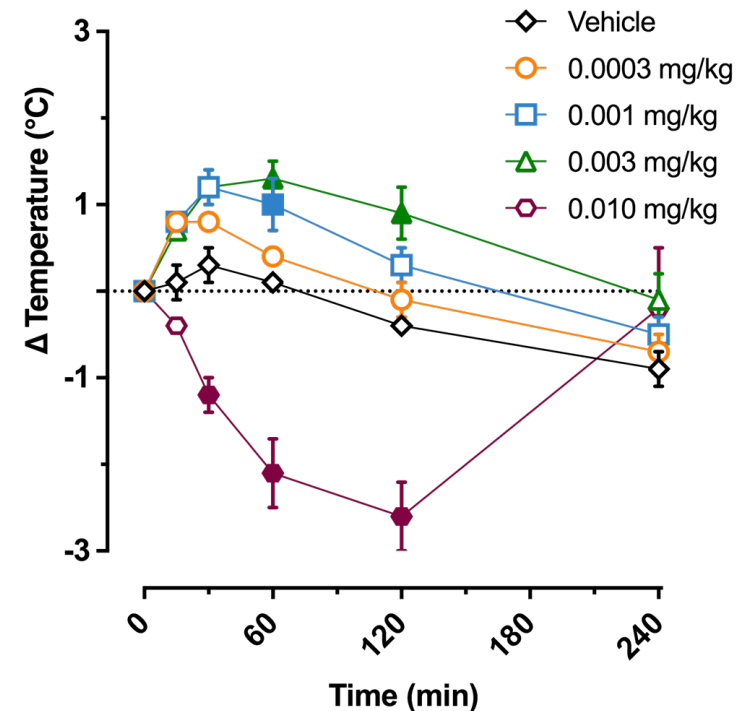
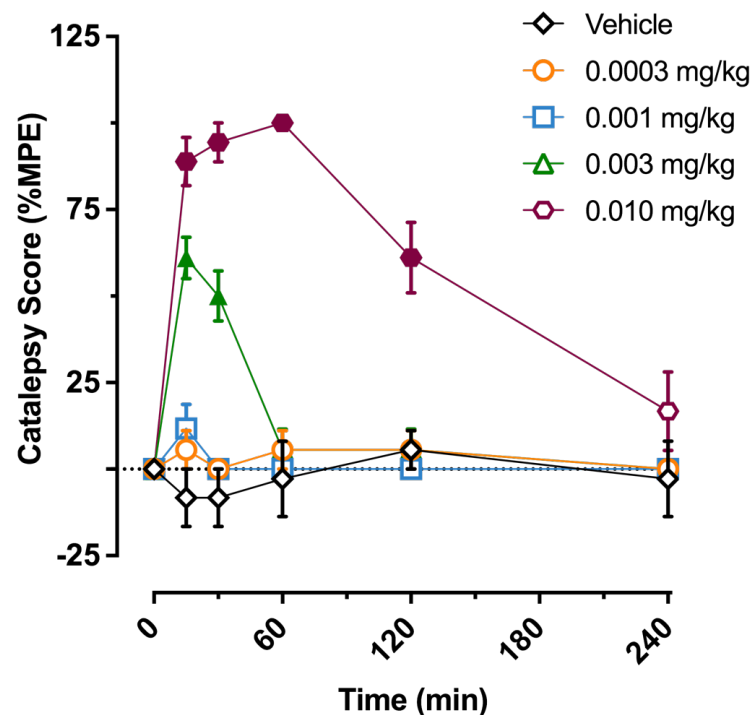
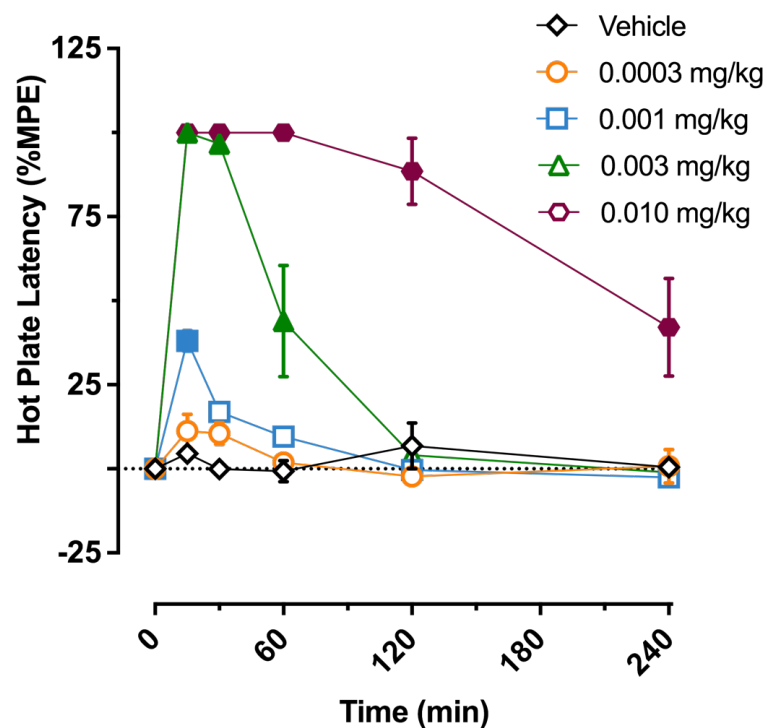
- Potency ( $EC_{50}$ ): 0.348 nM
  - 95% Confidence Interval: 0.137 – 0.876 nM
  - Comparable to etonitazene
  - 800x greater than morphine, 40x greater than fentanyl
- Efficacy ( $E_{max}$ ): 298%
  - 95% Confidence Interval: 264% – 333%
  - Comparable to fentanyl
  - 2x greater than morphine



## Pharmacodynamic Studies

- Hot Plate Latency: dose-dependent increases observed
  - Antinociceptive effect (~10x greater than fentanyl)
  - Maximum cut-off reached for the two highest doses
- Catalepsy: dose-dependent catalepsy observed at two highest doses
  - Immobility, splayed limbs, flattened posture
- Temperature: observed significant temperature changes
  - Small increases at low dose
  - Sustained decreases at high dose

# Pharmacodynamic Studies







# Toxicological Analysis

# Standard Addition Methodology

## Sample Preparation

- Standard Addition
  - Four total aliquots
  - Up-spikes at 0.2, 2, 20 ng/mL
- Internal Standard
  - Fentanyl-D5
- Basic Liquid-Liquid Extraction
  - 0.1 M Borax Buffer, pH 10.4
  - 70:30 *N*-butyl chloride:ethyl acetate



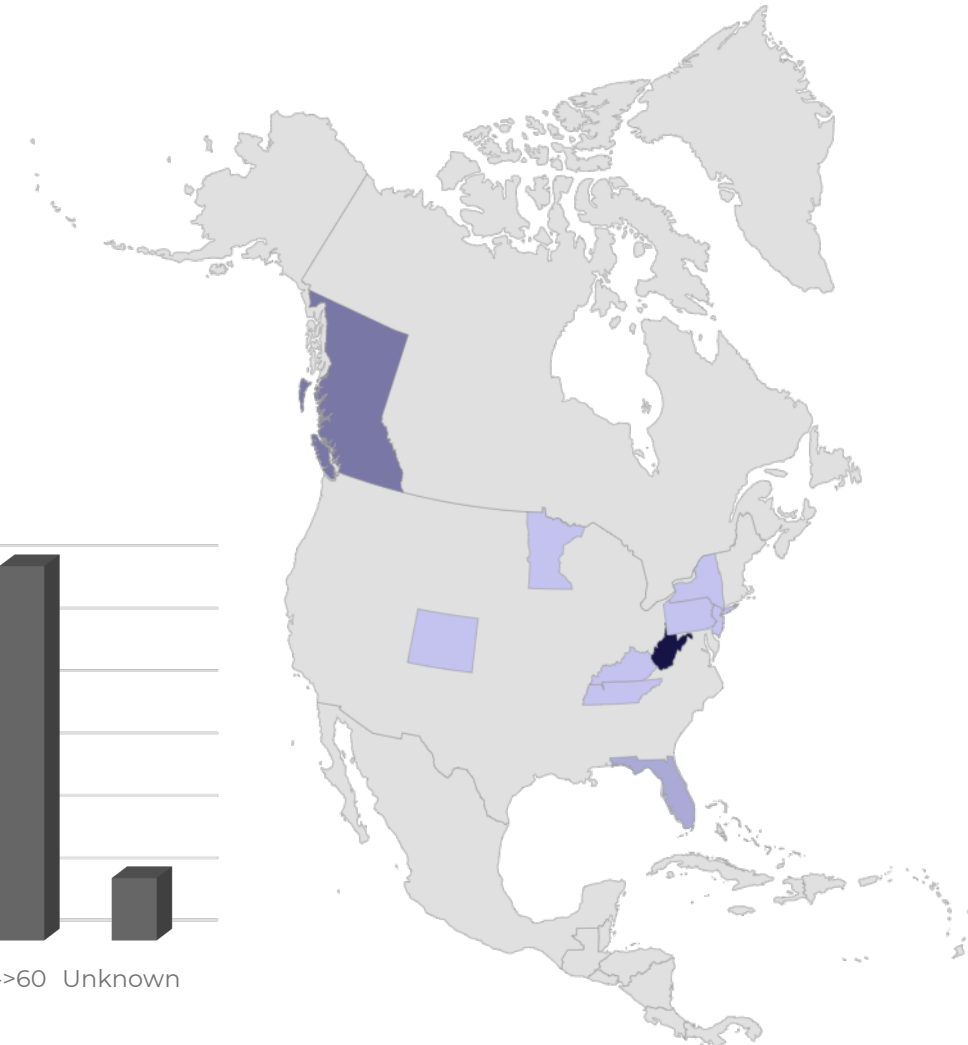
## Instrumental Analysis

- Waters Xevo TQ-S Micro LC-MS/MS
- Mobile phase compositions:
  - 0.1% Formic Acid in Water
  - 0.1% Formic Acid in Methanol
- Analytical Column
  - Agilent InfinityLab Poroshell 120 EC-C18 3.0 x 100mm, 2.7 μm

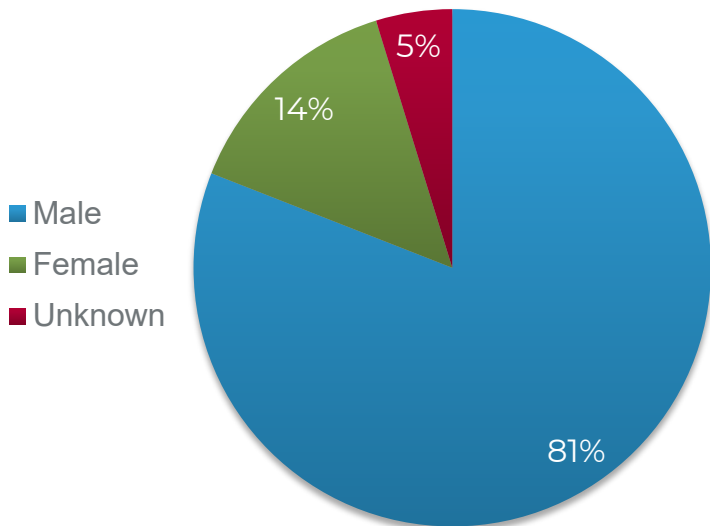
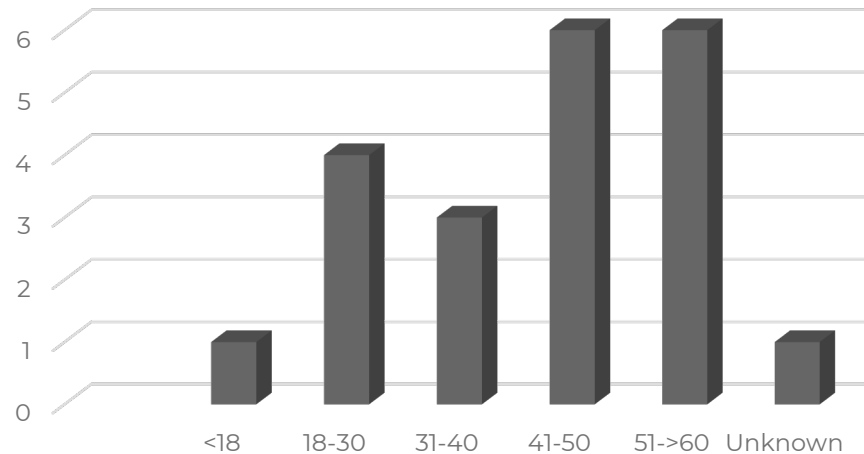
| Time (min) | Flow (mL/min) | %A | %B |
|------------|---------------|----|----|
| Initial    | 0.4           | 50 | 50 |
| 1.0        | 0.4           | 50 | 50 |
| 4.0        | 0.4           | 5  | 95 |
| 5.0        | 0.4           | 5  | 95 |
| 5.1        | 0.4           | 50 | 50 |
| 6.0        | 0.4           | 50 | 50 |

# Cases Received

- 21 cases submitted from medical examiner and coroner's offices
  - Collected January 2021 – October 2021
  - Collaboration with NMS Labs
- Cases from United States & Canada
  - All postmortem cases



Age Range

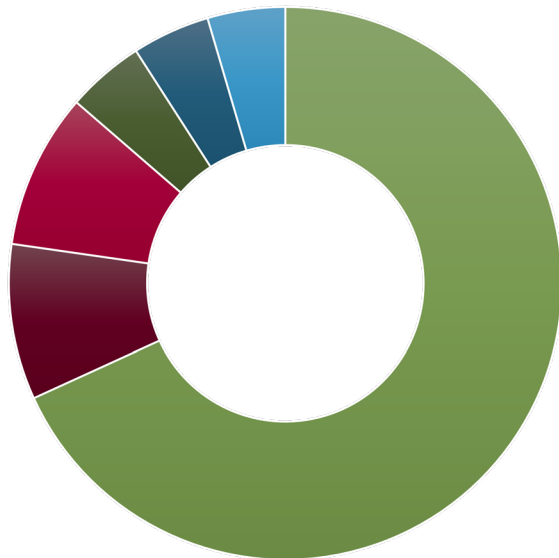




# Additional Case Information

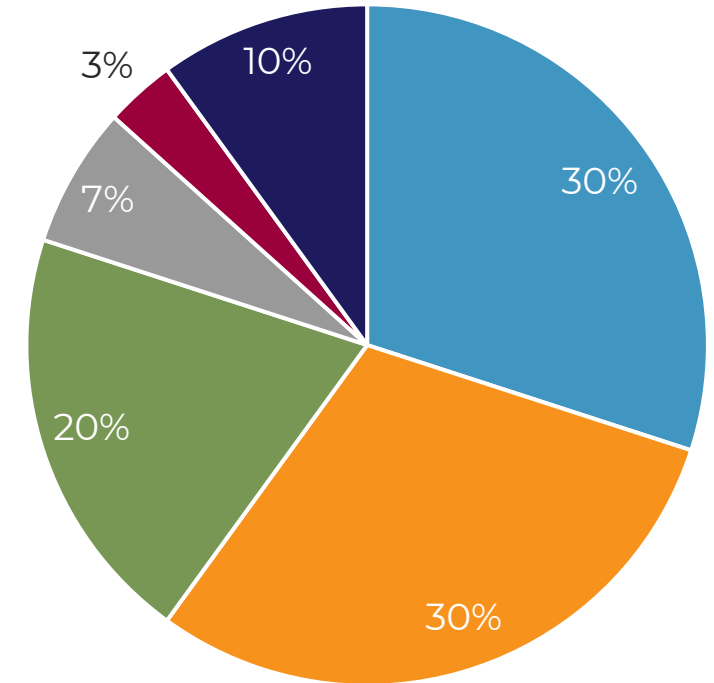
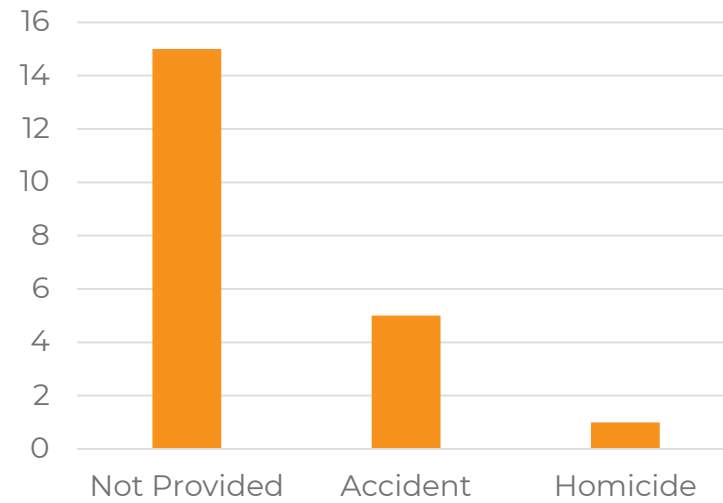
## Case Histories

Cause of Death



- Natural
- Not Provided
- N-Pyrrolidino Etonitazene Toxicity
- Multidrug Toxicity
- Multiple GSWs
- Combined Intoxication

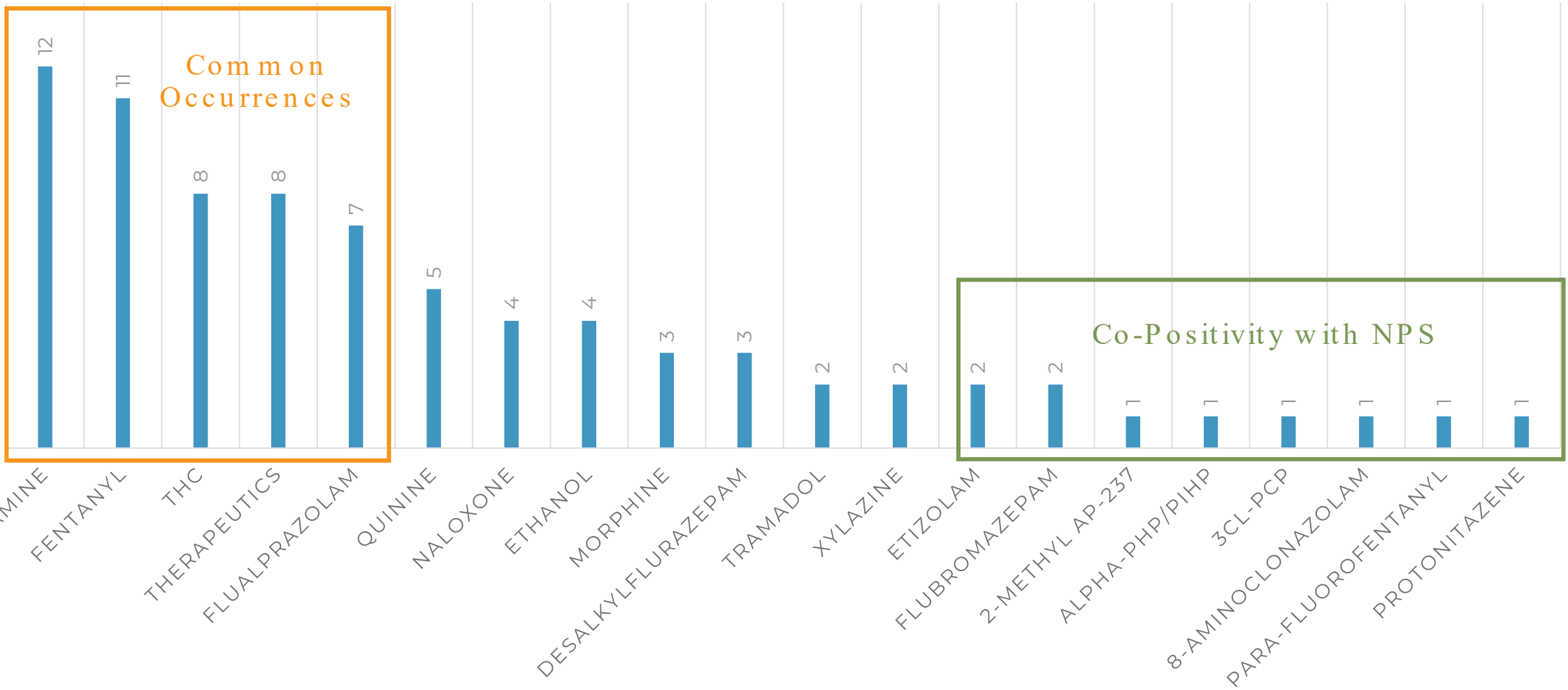
Manner of Death



- Found Unresponsive
- History of Drug Use
- Not Provided
- Prior Medical History
- Motor Vehicle Accident
- Suspected OD

# Qualitative Results

Screening performed at NMS Labs (LC-TOF-MS) and CFSRE (LC-QTOF-MS)



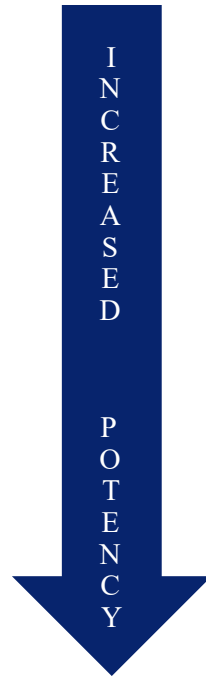
## Quantitative Results

- 15 out of 21 cases quantitated
  - 6 blood cases reported positive
  - 1 urine case quantitated

|                           | Matrix | N  | Mean ± Std. Dev. (ng/mL) | Median (ng/mL) | Range (ng/mL) |
|---------------------------|--------|----|--------------------------|----------------|---------------|
| N-Pyrrolidino Etonitazene | Blood  | 15 | 3.9 ± 5.9                | 2.4            | 0.3 - 25      |
|                           | Urine  | 1  | 1.5                      | N/A            | N/A           |

# Comparing Concentrations in Death Cases

*\*Order similar to reported in vitro potency*



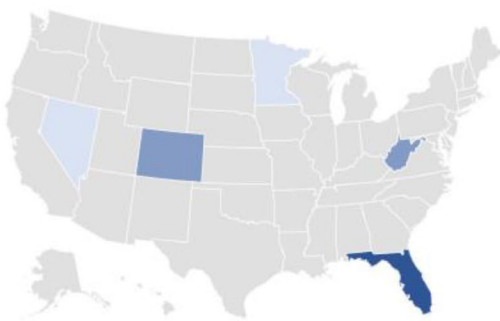
| Drug                       | N  | Mean ( $\pm$ SD)<br>(ng/mL) | Median<br>(ng/mL) | Range<br>(ng/mL) |
|----------------------------|----|-----------------------------|-------------------|------------------|
| Etodesnitazene             | 15 | 40 $\pm$ 61                 | 5.2               | 0.53 - 230       |
| Protonitazene*             | 3  | 11 $\pm$ 9.9                | 5                 | 3.1 - 25         |
| Metonitazene               | 18 | 6.3 $\pm$ 7.5               | 3.8               | 0.5 - 33         |
| Butonitazene               | 1  | 3.2                         | N/A               | N/A              |
| N-Pyrrolidino Etonitazene* | 15 | 3.9 $\pm$ 5.9               | 2.4               | 0.3 - 25         |
| Isotonitazene*             | 69 | 1.59 $\pm$ 1.81             | 1.0               | 0.5 - 9          |

2021

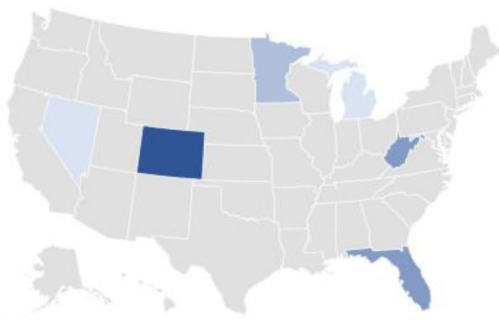
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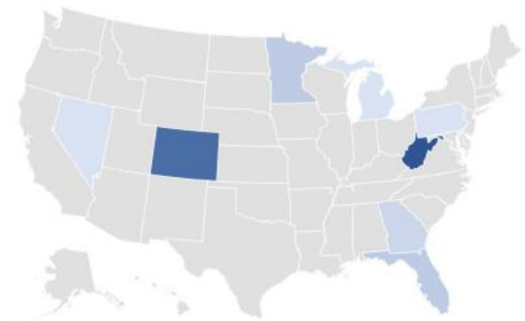
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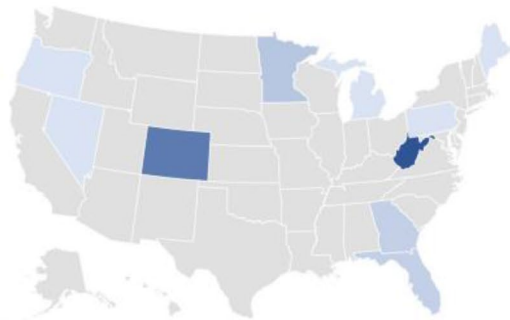
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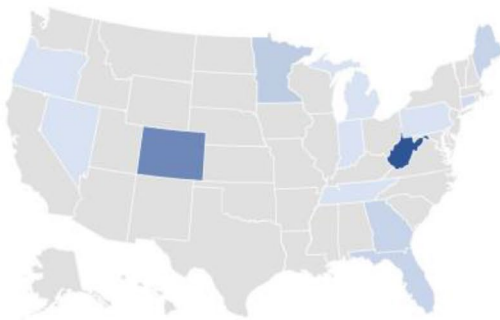
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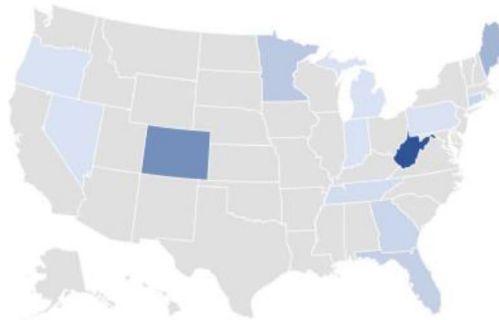
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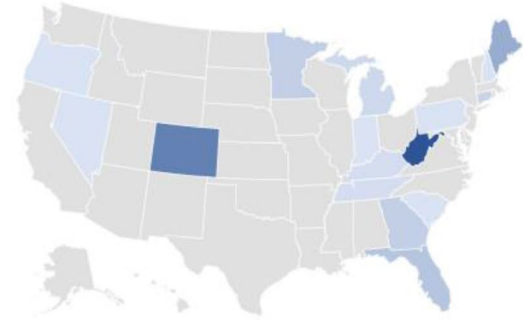
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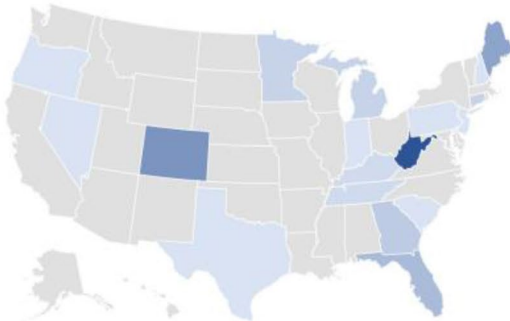
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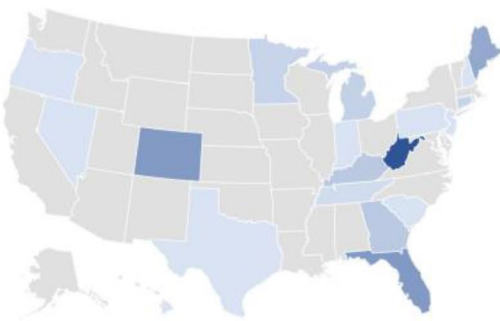
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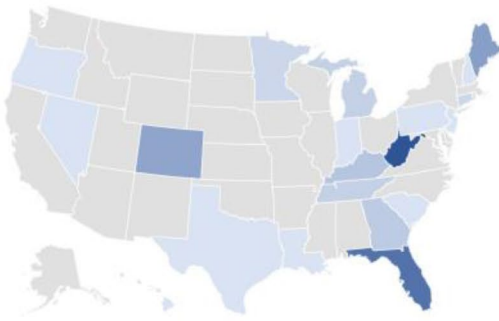
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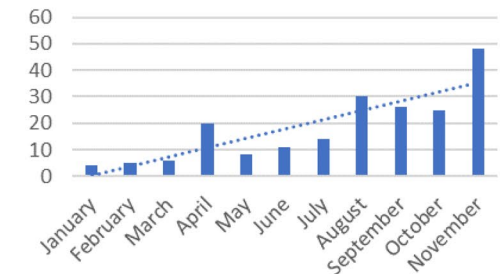
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November



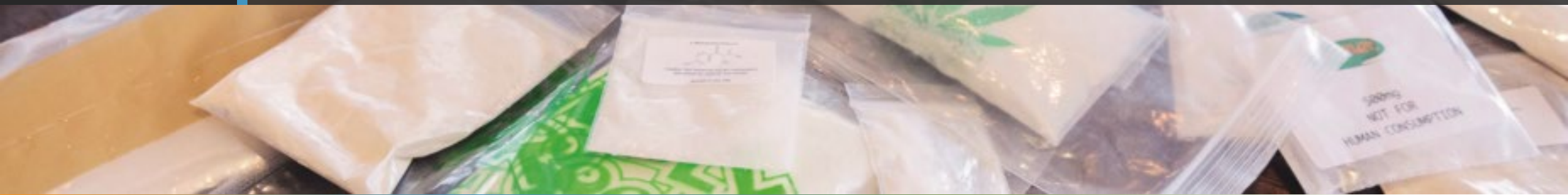
Cases Per Month







## Case Examples



## Case 1

|                           | Matrix        | Concentration (ng/mL) | Other Results                                    |
|---------------------------|---------------|-----------------------|--|
| N-Pyrrolidino Etonitazene | Femoral Blood | 1.7                   | Fentanyl (2.7 ng/mL), 4-ANPP, Caffeine, Cotinine |

- Case History:
  - 46 y/o male with a history of obesity and “heroin” use
- Case Details:
  - Femoral blood collected 10/4/2021
  - Tennessee, USA
- Manner of Death: Accidental
- Cause of Death: Combined *N-pyrrolidino etonitazene* and fentanyl intoxication. Secondary conditions included obesity and cardiomyopathy.

## Case 2

|                           | Matrix        | Concentration (ng/mL) | Other Results   |
|---------------------------|---------------|-----------------------|---|
| N-Pyrrolidino Etonitazene | Femoral Blood | 2.5                   | Ethanol (0.55 g/L), THC (9.6 ng/mL), THC-OH, THC-COOH, 7-Aminoclonazepam, Caffeine, Venlafaxine |
|                           | Urine         | 1.5                   |   |

- Case History:

- 26 y/o male found face down in his bedroom. There was a plate with a white powdered substance organized in lines. No needles, tubes, or pipes were found. History of marijuana and tobacco use.

- Case Details:

- Femoral blood and urine collected 4/2/2021
  - Minnesota, USA

- Manner of Death: Accidental

- Cause of Death: Toxic effects of **N-pyrrolidino etonitazene**





# Conclusion



## Conclusion

- Paired *in vivo* and *in vitro* characterization of *N*-pyrrolidino etonitazene shows that this new synthetic opioid:
  - Extremely potent MOR agonist
  - High MOR activation potential; comparable to etonitazene
  - Produced significant analgesic effects
- Quantitation of *N*-pyrrolidino etonitazene in blood indicated low ng/mL concentrations
  - Sensitive methodology necessary
  - Only opioid in 7 cases (33%)
  - Found alongside novel benzodiazepines (benzo-dope)
  - Also discovered with stimulants, opioids, and many other NPS



## Published Work


Archives of Toxicology

<https://doi.org/10.1007/s00204-022-03276-4>

ORGAN TOXICITY AND MECHANISMS



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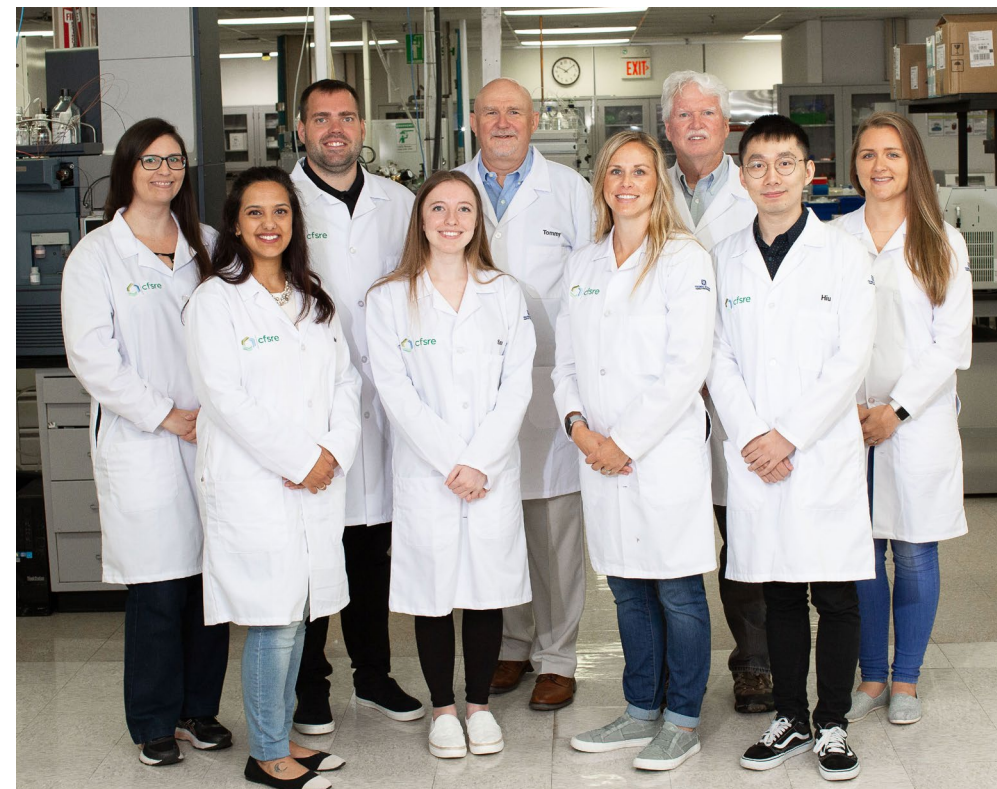
- Co-Authors

- Marthe Vandeputte and Christophe Stove – Ghent University
- Mike Baumann, Donna Walther, and Grant Glatfelter – Designer Drug Research Unit at NIDA
- Alex Krotulski and Barry Logan – CFSRE
- Donna Papsun – NMS Labs

- CFSRE Staff

- NMS Labs

- Collaborations with medical examiner and coroner offices





Thank you!

Questions?

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