Solid Phase Extraction of Novel Synthetic 2-Benzylbenzimidazole Opioid Compounds "Nitazenes"

UCT Part Numbers

CSDAU133
Clean Screen® DAU
3 mL, 130 mg

SPHPO7001-10
Select pH buffer pouch 100mM
Phosphate buffer pH 7.0

SCS27-C181021
SelectraCore® C18 Column
100 x 2.1 mm, 2.7 µm

SCS27-C18GDC21
SelectraCore® C18 Guard Column
5 x 2.1 mm, 2.7 µm

SLGRDHLDR-HPOPT
Selectra® Direct Connect Guard
Holder

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Summary:

A new class of synthetic opioids is emerging called benzylbenzimidazole-opioids also known as "nitazenes". Nitazene compounds were first synthesized in the 1950s as a potential analgesic.[1] These compounds were never approved for clinical use, but they are re-emerging as a new threat in the ongoing opioid epidemic. These potent synthetic opioids range from three to twenty times more potent than fentanyl.[2] Isotonitazene was the first nitazene analog detected in biological samples in the United States by the CFRSE in July 2019.[3] The number of cases across the country continues to rise. In total eight nitazenes have been temporarily added to schedule I by the Drug Enforcement Administration (DEA).[4] This application note introduces a simple targeted extraction method for the analysis of nine nitazene compounds from urine and blood utilizing UCT's flagship Clean Screen® DAU column and new SelectraCore® C18 core-shell column.

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Sample Pretreatment:

In a test tube add 0.5 mL of sample, internal standard, 200 µL of acetonitrile (ACN), and 1.3 mL of 100 mM phosphate buffer pH 7. Vortex and centrifuge samples for 10 minutes at 3000 rpm.

SPE Procedure:

1. **Condition Column:**

   1 x 3 mL methanol (MeOH)

   1 x 3 mL phosphate buffer pH 7

2. **Load Sample:**

   Load at 1 to 2 mL/minute

3. **Wash Column:**

   1 x 3 mL deionized water

   1 x 3 mL 50:50 MeOH:H_2O

4. **Dry Column:**

   Dry column for at least 10 minutes under full pressure or vacuum

5. **Elute:**

   1 x 3 mL of MeOH:NH_4OH (98:2)

   Note: prepare elution solvent daily

6. **Evaporate:**

   Add 250 µL of 10% HCl in methanol and vortex

   Evaporate eluate at 10 psi, 35°C

7. **Reconstitute:**

   1 mL of 50:50 MeOH:H_2O

Notes:

- Centrifuging blood samples causes a decrease in sample recovery, but improves visual cleanliness of sample at the end of SPE
- As an alternative, samples can be evaporated at 30°C, 5 psi

LC-MS/MS Parameters:

LC-MS/MS System: Shimadzu Nexera LC-30AD with MS-8050
UHPLC Column: SelectraCore® C18 Column 100 x 2.1 mm, 2.7 µm (PN: SCS27-C18021)
Guard Column: SelectraCore® C18 Guard Column 5 x 2.1 mm, 2.7 µm (PN: SCS27-C18GDC21)
Column Temperature: 40°C
Flow Rate: 0.45 mL/min
Injection Volume: 5 µL
Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: 0.1% formic acid in methanol

Gradient Program:

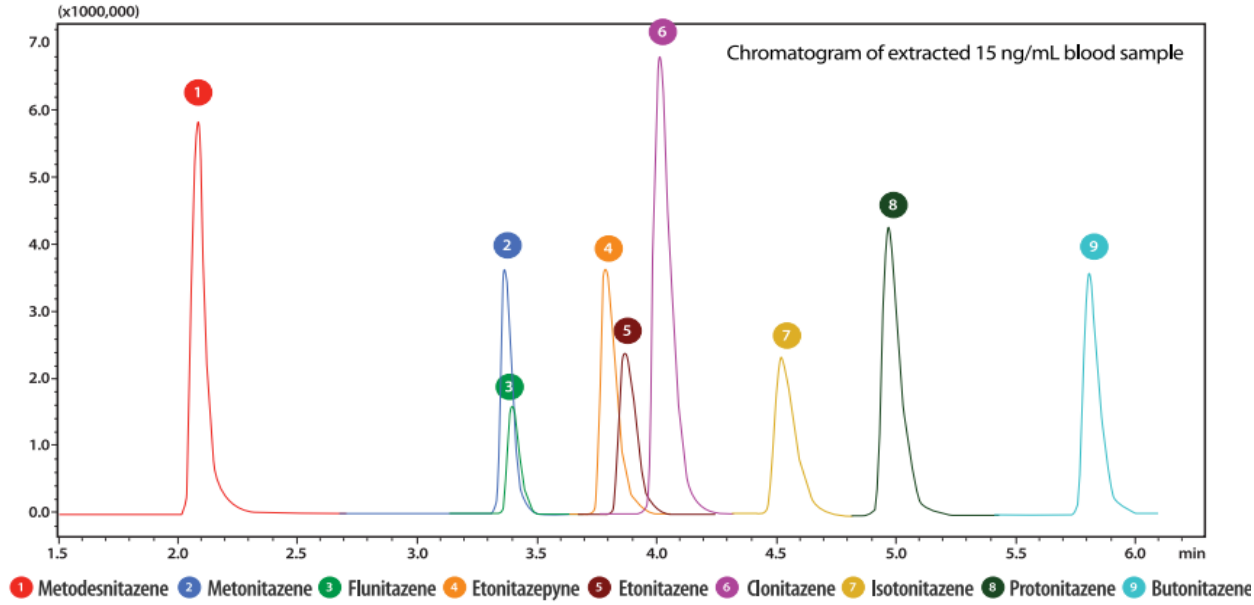
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	90	10
2.5-3.5	57	43
7	30	70
8-11	0	100
11.3-15	90	10

MRM Table:

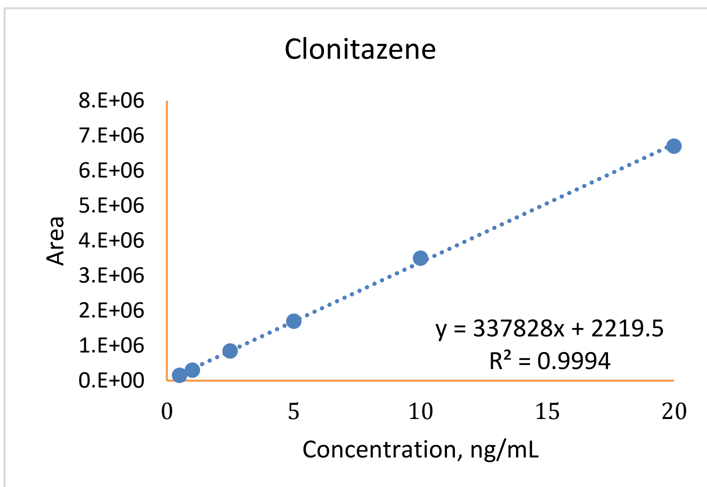
Analyte	Parent Ion (m/z)	Product Ion 1 (m/z)	CE (eV)	Product Ion 2 (m/z)	CE (eV)	RT (min)
Butonitazene	425.5	100.1	-23	72.1	-45	5.83
Clonitazene	386.5	100.1	-26	125.1	-36	4.03
Etonitazene	397.4	100.1	-21	72.0	-36	3.88
Etonitazepyne	395.6	98.1	-23	56.1	-55	3.80
Flunitazene	371.3	100.1	-23	73.1	-26	3.41
Isotonitazene	411.5	100.1	-21	72.2	-45	4.53
Metodesnitazene	339.2	100.1	-21	72.1	-40	2.09
Metonitazene	383.5	100.1	-22	72.2	-39	3.38
Protonitazene	411.7	100.1	-24	72.1	-39	4.98

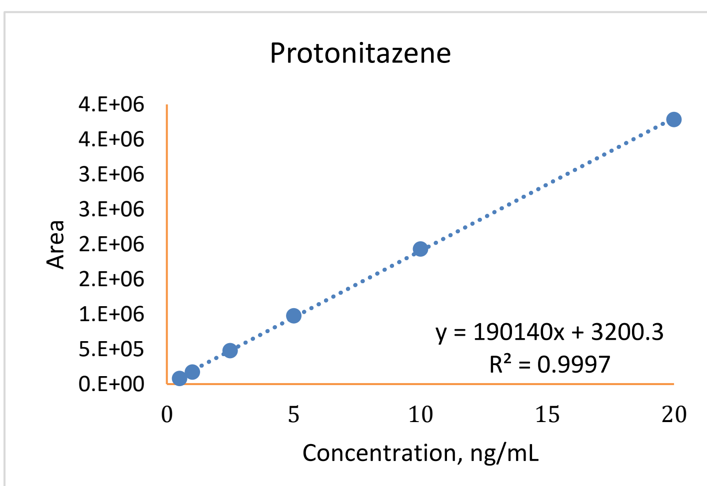
*CE = collision energy, RT = retention time

Chromatogram:



Example Solvent Calibration Curves:





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Results:

Urine (n=5)

Analyte	1 ng/mL			5 ng/mL			15 ng/mL		
	% Recovery	% RSD	Matrix Effect	% Recovery	% RSD	Matrix Effect	% Recovery	% RSD	Matrix Effect
Butonitazene	97	4	-14%	93	2	-12%	93	2	-12%
Clonitazene	101	6	-5%	93	2	0%	95	2	-3%
Etonitazene	103	6	-5%	94	1	-1%	99	1	-6%
Etonitazepyne	104	6	-2%	95	1	4%	96	3	-1%
Flunitazene	102	5	-5%	100	4	1%	98	2	-2%
Isotonitazene	100	4	-4%	94	2	1%	98	2	-1%
Metodesnitazene	98	2	8%	93	4	5%	106	4	-10%
Metonitazene	97	3	0%	92	2	3%	98	1	-4%
Protonitazene	100	4	-5%	94	0	1%	96	1	-3%

Blood (n=5)

Analyte	1 ng/mL			5 ng/mL			15 ng/mL		
	% Recovery	% RSD	Matrix Effect	% Recovery	% RSD	Matrix Effect	% Recovery	% RSD	Matrix Effect
Butonitazene	75	5	9%	74	6	14	81	3	0%
Clonitazene	87	3	6%	83	5	12	89	3	-3%
Etonitazene	93	4	0%	87	5	8	94	1	-5%
Etonitazepyne	94	2	13%	89	4	18	97	1	0%
Flunitazene	96	5	-3%	92	3	5	98	2	-11%
Isotonitazene	85	4	8%	83	5	15	90	4	-1%
Metodesnitazene	95	6	11%	89	4	17	94	6	-6%
Metonitazene	95	4	8%	89	4	16	98	3	-1%
Protonitazene	87	4	3%	81	3	10	87	3	-5%

Recovery was calculated by comparing peak area of pre-spiked samples to peak area of post-spiked samples. Matrix effects were calculated by comparing peak area of post-spiked samples to peak area of evaporated solvent standards. A negative matrix effect indicates ion suppression while a positive matrix effect indicates ion enhancement.

Conclusion/Discussion:

A simple extraction method was developed for the extraction of nine nitazene compounds from urine and blood. Analytes were extracted using UCT's flagship column Clean Screen® DAU and analyzed on a LC-MS/MS equipped with UCT's new SelectraCore® C18 core-shell column. All analytes were separated in 6 minutes with a short total run time of 15 minutes. Isomers protonitazene and isotonitazene were successfully separated on the core-shell column. Due to these compounds' novelty and potency, developing an extraction with a low limit of quantitation was crucial and challenging.

A sizeable amount of the non-polar analytes, particularly butonitazene and isotonitazene, remain in the test tube after loading the sample onto the SPE cartridge. To better retain the analytes, 200 µL of acetonitrile was added during sample preparation. This is vital for detection and quantitation at low concentrations. Another discovery made during method development was that free-base nitazene compounds are volatile. It was difficult to avoid the evaporation step after extraction as these compounds are present at low concentrations in biological samples. Like amphetamines, hydrochloride acid was added to the elution solvent before evaporation to create more stable salt forms.

The extraction method was evaluated using quality control samples prepared at low, medium, and high concentrations. Recovery and matrix effect for each analyte were calculated using pre-spiked samples, post-spiked samples, and evaporated solvent standards. Pre-spiked samples are extracted biological samples spiked during sample preparation. Post-spiked samples are extracted biological samples spiked after the extraction into the elution solvent. Evaporated solvent standards are spiked elution solvent samples with 10% HCl that were fully dried and reconstituted. Formulas for recovery and matrix effect are shown below:

$$\%\ Recovery = \frac{Peak\ Area\ of\ Pre-Spiked\ Samples}{Peak\ Area\ of\ Post-Spiked\ Samples} \times 100$$

$$Matrix\ Effect = \left(\frac{Peak\ Area\ of\ Post-Spiked\ Samples}{Peak\ Area\ of\ Evaporated\ Samples}\right) \times 100$$

Extraction recoveries of analytes from urine ranged from 93-106% with relative standard deviations less than 10%. Matrix effects for all analytes were within ± 25% making it easy to implement and validate this method in laboratories that follow ANSI/ASB Standard 036. Extraction recoveries of analytes from blood ranged from 74-96% with relative standard deviations less than 10%. Matrix effects for all analytes in blood were also within ± 25%.

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References:

1. Diversion Control Division, Benzimidazole-Opioids Other Name: Nitazenes (2022).
2. Vandeputte, M.M., Krotulski, A.J., Walther, D. *et al.* Pharmacological evaluation and forensic case series of *N*-pyrrolidino etonitazene (etonitazepyne), a newly emerging 2-benzylbenzimidazole 'nitazene' synthetic opioid. *Arch Toxicol* **96**, 1845–1863 (2022). https://doi.org/10.1007/s00204-022-03276-4
3. Alex J Krotulski, Donna M Papsun, Sherri L Kacinko, Barry K Logan, Isotonitazene Quantitation and Metabolite Discovery in Authentic Forensic Casework, *Journal of Analytical Toxicology*, Volume 44, Issue 6, July 2020, Pages 521–530, https://doi.org/10.1093/jat/bkaa016
4. Seven Benzimidazole-Opioids: Butonitazene, Etodesnitazene, Flunitazene, Metodesnitazene, Metonitazene, N-Pyrrolidino Etonitazene, and Protonitazene, 86 Fed. Reg. 69183-69186 (December 7, 2021)

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