LC-QTOF-MS Method Development and Validation for the Screening of Nitazene Analogs in Whole Blood

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INTRODUCTION

Nitazene analogs have become an increasing concern for the forensic toxicology community since their emergence in 2019. Similar to fentanyl analogs, nitazenes produce opioid-like effects and exhibit high potencies. The rapidly changing landscape of forensically relevant nitazenes can make it difficult to screen for nitazene compounds using traditional screening techniques such as immunoassay. Due to the development and manufacturing time needed for creating new immunoassay kits, there is a potential to produce false negative results when analyzing novel compounds.

Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) has been increasingly utilized by laboratories for novel drug screening (1). LC-QTOF-MS allows new compounds to be easily added to existing libraries, making integration with current methods seamless (2). Additionally, LC-QTOF-MS can offer more selectivity and sensitivity over immunoassay when available.

This research aimed to develop and validate a LC-QTOF-MS method using Targeted MS/MS for 7 forensically relevant nitazene analogs (4'-OH nitazene, 5-methyl etodesnitazene, isotonitazene, metodesnitazene, N-piperidinyl etonitazene, N-pyrrolidino etonitazene, protonitazene) and two internal standards (isotonitazene $^{13}C_6$ and metodesnitazene-D_4). This method is intended to be used as baseline for future research on nitazene analysis.

ACKNOWLEDGEMENTS

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RESOURCES

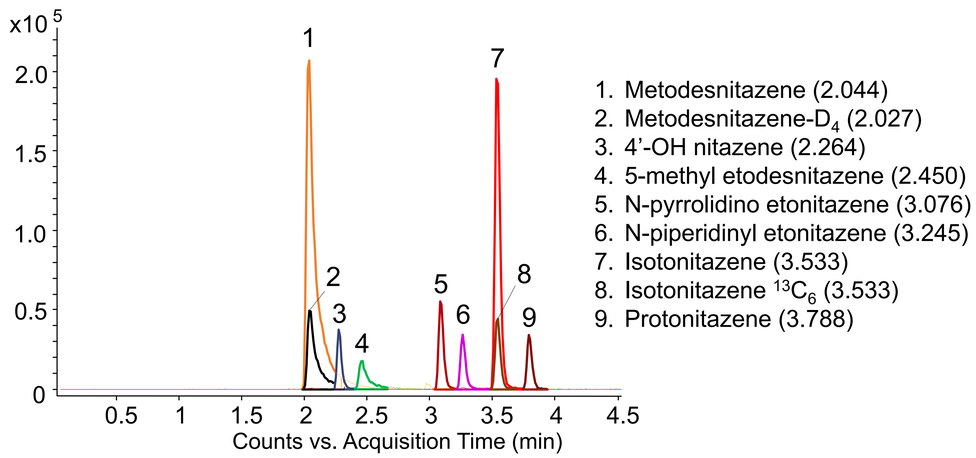
1. Krotulski, A.J., Papsun, D.M., Kacinko, S.L. and Logan, B.K. (2020) Isotonitazene Quantitation and Metabolite Discovery in Authentic Forensic Casework. *Journal of Analytical Toxicology*, **44**, 521–530.
2. Griswold, M.K., Chai, P.R., Krotulski, A.J., Friscia, M., Chapman, B.P., Varma, N., et al. (2017) A Novel Oral Fluid Assay (LC-QTOF-MS) for the Detection of Fentanyl and Clandestine Opioids in Oral Fluid After Reported Heroin Overdose. *Journal of Medical Toxicology*, **13**, 287–292.

RESULTS & DISCUSSION

Table 1: LC Gradient

Time	%B
0.00	10
0.25	10
1.00	25
1.75	35
4.75	50
5.00	90
7.00	90
7.10	10
10.00	10

Figure 1: Chromatography with RT (mins)- Unextracted sample, 5 ng/mL



1. Metodesnitazene (2.044)
2. Metodesnitazene-D_4 (2.027)
3. 4'-OH nitazene (2.264)
4. 5-methyl etodesnitazene (2.450)
5. N-pyrrolidino etonitazene (3.076)
6. N-piperidinyl etonitazene (3.245)
7. Isotonitazene (3.533)
8. Isotonitazene $^{13}C_6$ (3.533)
9. Protonitazene (3.788)

Table 2: MS/MS Parameters and Validation Results

Analyte	M+H$^+$ (Da)	Collision Energy (eV)	Retention Time (min)	Limit of Detection (ng/mL)	Matrix Effects (%)	
					5 ng/mL	25 ng/mL
4'-OH nitazene	369.1921	38	2.264	5.0	-40	-51
5-methyl etodesnitazene	366.2539	34	2.450	5.0	41	-25
Isotonitazene	411.2390	42	3.533	1.0	-30	-47
Metodesnitazene	338.2226	36	2.044	5.0	-21	-42
N-piperidinyl etonitazene	409.2234	70	3.245	5.0	-50	-59
N-pyrrolidino etonitazene	395.2077	48	3.076	1.0	-17	-38
Protonitazene	411.2390	40	3.788	1.0	-26	-48
Isotonitazene $^{13}C_6$	342.2477	42	2.027	-	-31	-48
Metodesnitazene-D_4	417.2591	36	3.533	-	-11	-38

Table 3: ESI+ Source Conditions

Sheath gas temperature	400 ˚C
Sheath gas flow	12 L/min
Nebulizer pressure	30 psi
Capillary voltage	4000 V
Nozzle voltage	0 V
Drying gas temperature	350 ˚C
Drying gas flow	13 L/min

Table 4: Additional Validation Parameters

- No interferences were observed for any analyte
- No carryover observed at 100 ng/mL
- All analytes were stable for at least 48 hours on the autosampler (35˚C)
- Extended LOD studies were conducted using an additional 6 matrices (9 total) to demonstrate that LODs are not impacted by matrix effects

MATERIALS AND METHODS

Extraction procedure:

- Fortify 500 µL blank blood or sample with analyte mix solution, vortex
- Add 25 µL ISTD mix (20 ng/mL final)
- Add 50 µL NH4OH
- Add 1 mL 10 mM borate buffer (pH 9.1)
- Add 3 mL *N*-butyl chloride
- Rotate 15 mins
- Centrifuge 10 mins @4000 rpm
- Transfer organic layer to a conical tube
- Dry at 40˚C for ~15 min until dry
- Reconstitute in 200 µL starting mobile phase conditions

Instrument Conditions:

Separation

- Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 100 mm x 2.7 µm) column with matching guard column
  - Gradient elution was used **(Table 1)**.
- *Mobile phase A:* 0.1% formic acid with 5 mM ammonium formate in deionized water
- *Mobile phase B*: 0.1% formic acid in ACN

Instrumentation

- Agilent 1290 Infinity LC & Agilent 6530 Accurate-Mass Q-TOF used for analysis
- Positive electrospray ionization mode used with optimized conditions **(Table 3)**.

CONCLUSIONS

- A liquid-liquid extraction with subsequent LC-QTOF-MS detection for nitazene analogs was successfully developed and validated to ASB 036 **(Table 2, Table 4)**.
- 5-methyl etodesnitazene was included in this method, which has not been included in previously published methods
- Isomers protonitazene and isotonitazene were baseline resolved with the developed gradient **(Figure 1)**.

DISCLOSURE

The authors declare no conflicts of interest or financial disclosures.