A Stability Assessment of Nitazenes in Aqueous Solutions for the Development of Sample Preparation Procedures

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INTRODUCTION

Nitazene opioids have become more prevalent in seized drug and toxicological samples over the last several years[1,2]. In response, more laboratories have developed analytical methods to detect these compounds, but new analytes continue to emerge. Sample preparation is a crucial step in the method development process and frequently involves adjusting pH with various reagents and buffers to isolate analytes from matrix components. Limited information is available about nitazene properties, including stability in various aqueous environments. Therefore, it is important to consider how pH adjustments could affect analyte stability, which could aid in new method development. To address this need, this study evaluated the stability of seven nitazenes in common extraction buffers and extreme pH solutions. The analytes tested were 4'-OH nitazene, 5-methyl etodesnitazene, isotonitazene, metodesnitazene, *N*-piperidinyl etonitazene, *N*-pyrrolidino etonitazene, and protonitazene (**Figure 1** and **Table 1**).

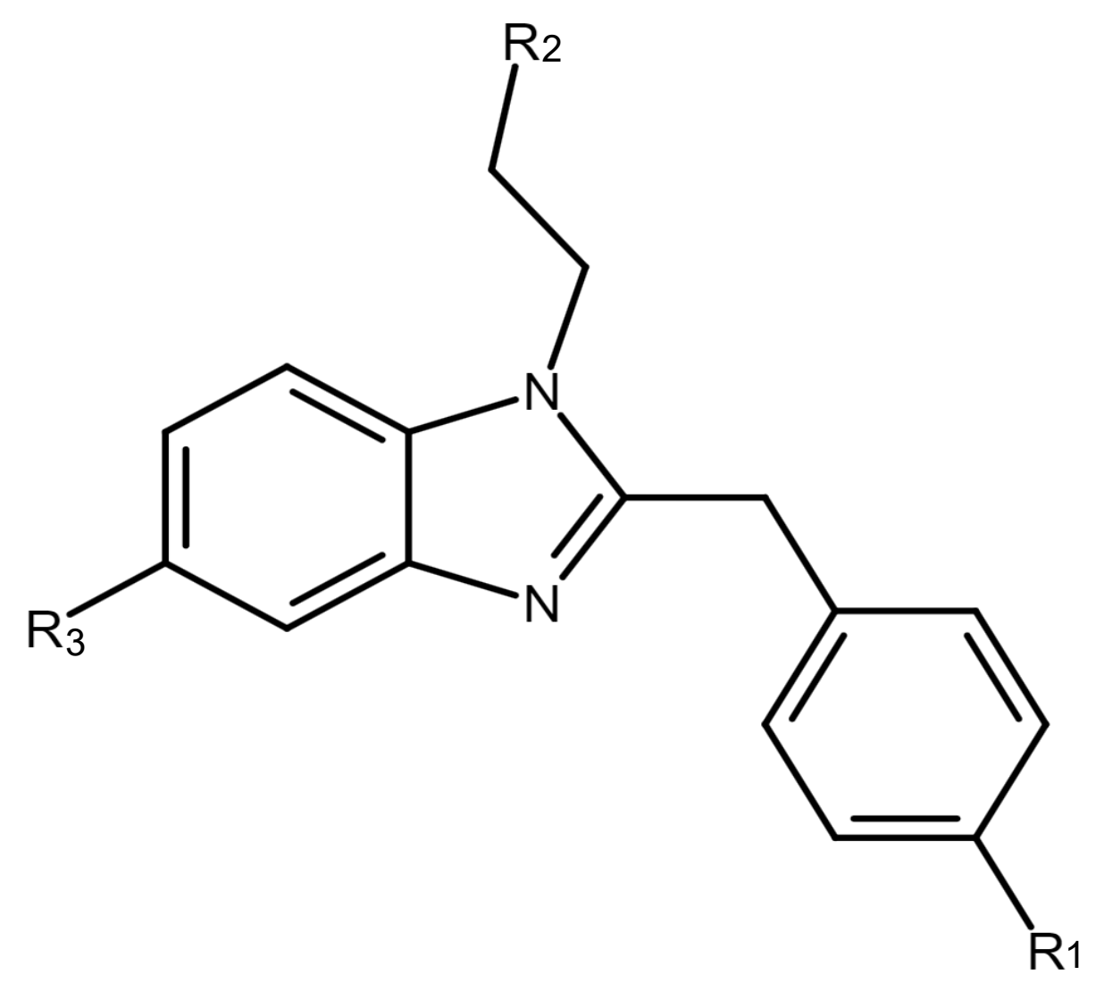

Figure 1. *Core structure of nitazenes*

Table 1. *Structural modifications of nitazenes included in this study*

Compound	R1	R2	R3
Metodesnitazene	CH_3O	$(CH_3CH_2)_2N$	H
4-OH nitazene	OH	$(CH_3CH_2)_2N$	NO_2
5-methyl etodesnitazene	CH_3CH_2O	$(CH_3CH_2)_2N$	CH_3
N-pyrrolidino etonitazene	CH_3CH_2O	$(C_4H_8)N$ (ring)	NO_2
N-piperidinyl etonitazene	CH_3CH_2O	$(C_5H_{10})N$ (ring)	NO_2
Isotonitazene	$(CH_3)_2CHO$	$(CH_3CH_2)_2N$	NO_2
Protonitazene	$CH_3CH_2CH_2O$	$(CH_3CH_2)_2N$	NO_2

DISCLOSURE

The authors have no conflicts to disclose.

ACKNOWLEDGEMENTS

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RESULTS & DISCUSSION

- Stability was evaluated by comparing mean peak area ratios at each timepoint to the mean at t_0 for each pH condition. Analytes were deemed unstable if the change in peak area ratio exceeded ±20%.
- Instability was observed for all analytes in 0.1 M ammonium hydroxide and in 0.05 M borate buffer (pH 9) and some instability was observed in 0.1 M phosphate buffer (pH 6).
- All analytes were stable ≥4 hours in 0.1 M HCl and ≥8 hours in 0.1 M sodium acetate buffer (pH 3.5) (**Figure 2**).
- Internal standards were able to compensate for instability (using peak area ratios) for up to 30 hours for all analytes except 4'-OH nitazene and 5-methyl etodesnitazene. Examples for deionized water and 0.05 M borate buffer (pH 9) are shown in **Figure 3**.

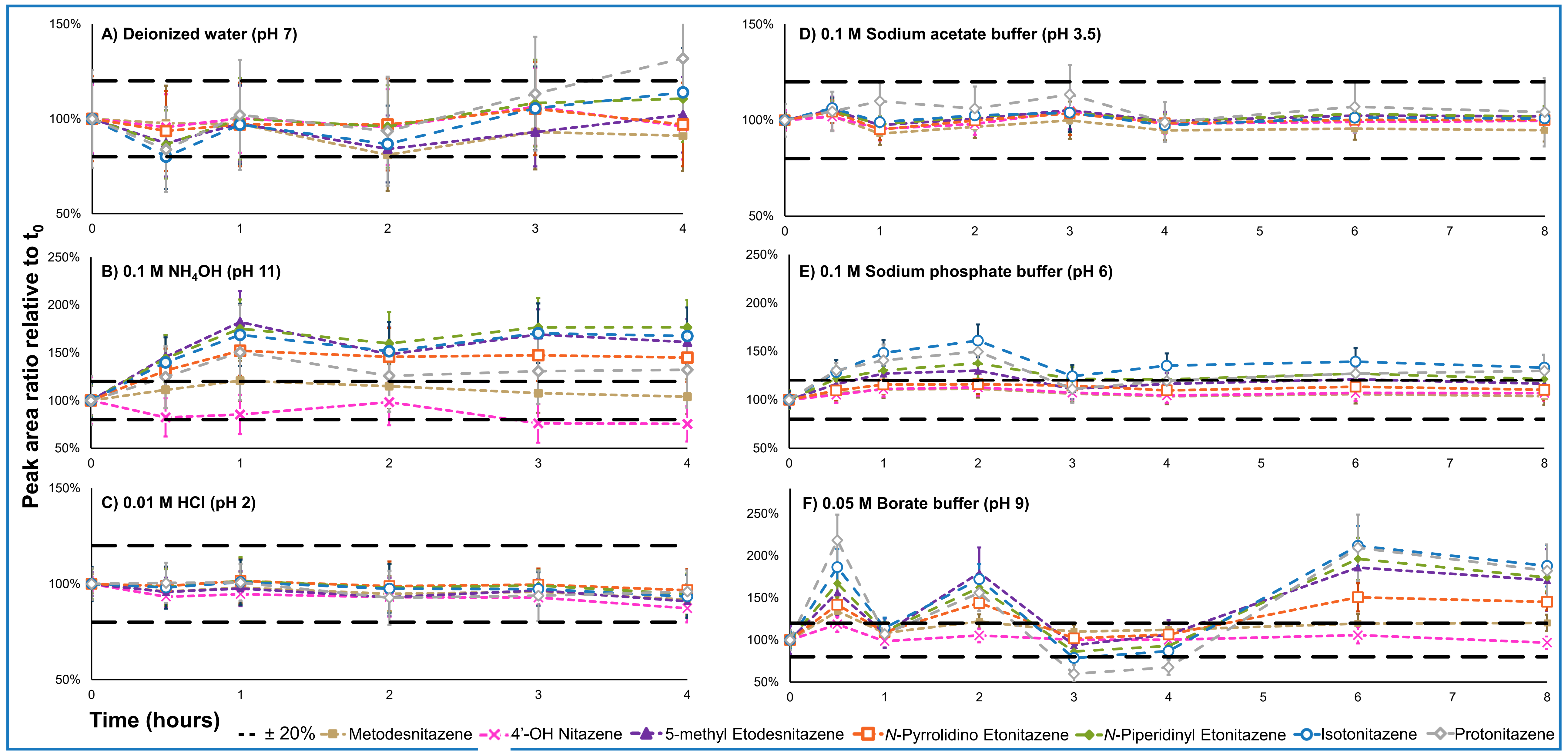

Figure 2. *Stability results relative to t_0 (±1SD) in each solvent (A-F). Analytes paired with metodesnitazene-D4 were metodesnitazene, 4'-OH nitazene, and 5-methyl etodesnitazene and remaining analytes were paired with isotonitazene-$^{13}C_6$. Stability thresholds were set at ±20% of t_0.*

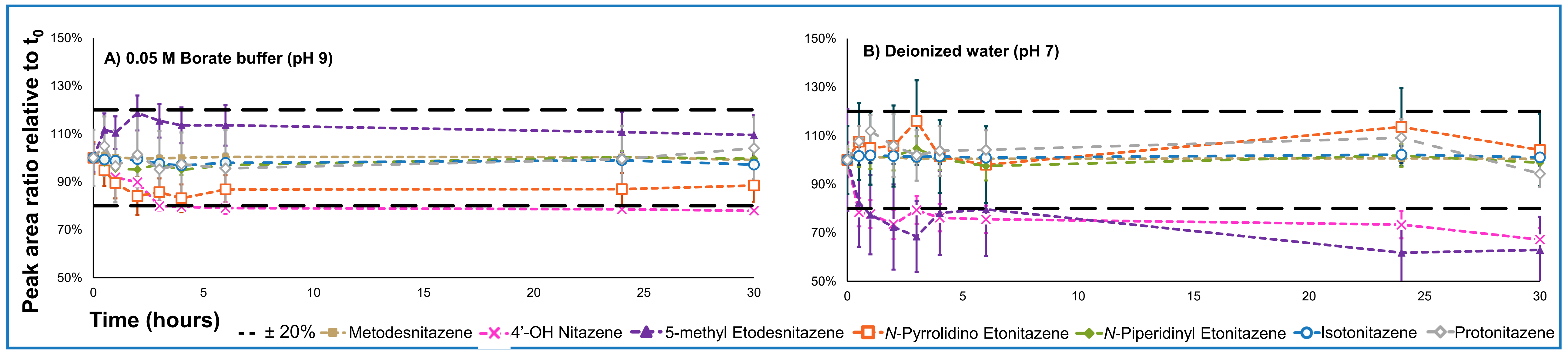

Figure 3. *Sample stability results with ISTD compensation for A) 0.05 M pH 9 borate buffer and B) deionized water.*

REFERENCES

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[2] Papsun, Donna M, Krotulski, Alex J, Logan, Barry K (2022). Proliferation of Novel Synthetic Opioids in Postmortem Investigations After Core-Structure Scheduling for Fentanyl-Related Substances. *Am J Forensic Med Pathol*, 43(4), 315-327; doi: 10.1097/PAF.0000000000000787.

MATERIALS & METHODS

- Solutions were prepared at 20 ng/mL in each solvent:

Solvent	pH
0.01 M hydrochloric acid (HCl),	pH 2
0.1 M sodium acetate buffer,	pH 3.5
0.1 M sodium phosphate buffer,	pH 6
Deionized water (H_2O),	~pH 7
0.05 M borate buffer,	pH 9
0.1 M ammonium hydroxide (NH_4OH),	pH 11

- Solutions were sampled in triplicate at set increments:
  - 0, 0.5, 1, 2, 3, 4 hours (H_2O, NH_4OH, and HCl)
  - 0-4, 6, 8 hours (all other buffers)
- Aliquots (50 µL) were fortified with internal standard (ISTD) then 20 µL was diluted with 180 µL mobile phase (90:10 A:B)
  - ISTD: 500 ng/mL metodesnitazene-D_4 & isotonitazene-$^{13}C_6$
- ISTD compensation was evaluated by analyzing aliquots (20 µL) containing both analyte (20 ng/mL) and ISTD (50 ng/mL) from t_0 (n=3) as described above
  - 0, 0.5, 1, 2, 3, 4, 6, 24, 30 hours
- All samples were analyzed using a previously validated LC-MS/MS method (**Table 2**)

Table 2. *Instrumental parameters*

Parameter	Value	
Instrument	Agilent 1290 Infinity II LC and Agilent 6470 Triple Quadrupole MS	
Column	Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm x 2.7 µm) with matching guard	
Mobile phase A	0.1% formic acid and 5 mM ammonium formate in H_2O	
Mobile phase B	0.1% formic acid in acetonitrile	
Column temperature	35 ºC	
Flow rate	0.4 mL/min	
Gradient (%B, time)	10% (hold) 25% (ramp) 35% (ramp) 50% (ramp) 90% (hold/wash) 10% (re-equilibration)	0.00 – 0.25 min 0.25 – 1.00 min 1.00 – 1.75 min 1.75 – 4.75 min 4.75 – 6.75 min 6.75 – 8.75 min
	9-minute total run-time	
Injection volume	5 µL	
Ionization	Electrospray ionization (positive mode)	
Acquisition	Multiple reaction monitoring (MRM)	

CONCLUSIONS

- Nitazene opioids can be stabilized by acidic conditions and destabilized by alkaline conditions in aqueous solution.
- Internal standards, especially paired isotopically labelled standards, can generally compensate for instability.
- These factors may be useful to consider during sample preparation method development.

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