

Highly sensitive MS/MS detection for confident identification of potent novel synthetic opioids and their metabolites

HRMS analysis of discarded authentic postmortem case samples using the SCIEX ZenoTOF 7600 system, powered by SCIEX OS software

Pierre Negri¹ and Alex J. Krotulski²

¹SCIEX, USA; ²Center for Forensic Science Research and Education at the Fredric Rieders Family Foundation, USA

The introduction of highly potent novel synthetic opioids (NSO) to the illicit drug market has been a major driver for the recent rise in the number of accidental drug overdoses. NSO are a class of novel psychoactive substances (NPS) that are commonly used as adulterants in heroin and counterfeit preparations to mimic the effects of controlled opioids. These substances vary greatly in potency and purity and thus often require only a small amount to cause acute intoxications. Their increasing occurrence in combined opioid drug toxicity cases, resulting in accidental and fatal drug overdoses, continues to create a major challenge for public health officials.

Traditionally, screening for ultra-potent substances was performed using targeted workflows, such as multiple reaction monitoring (MRM) using triple quadrupole mass spectrometers, because of the higher selectivity and sensitivity performance. However, the continuous emergence of NPS on the recreational drug market is creating an additional challenge for drug tracking agencies and laboratories to meet. High-resolution mass spectrometry has provided forensic toxicology laboratories with a unique tool for the untargeted detection and identification of these new emerging substances, with little or no method optimization necessary. In addition, accurate mass instruments are affording additional levels of certainty by reliably obtaining comprehensive MS/MS spectral fragment information that can be used for identification, confirmation, and/or library matching.



Figure 1: TOF MS/MS sensitivity gains using Zeno IDA for representative analytes. An average of ~9X gain in TOF MS/MS sensitivity was observed across all analytes identified in this study.



In this technical note, a highly sensitive method for the detection and identification of potent NSO in human whole blood is described. The technological enhancements of the ZenoTOF 7600 system¹ provide a high degree of sensitivity, selectivity and confidence for MS/MS experiments. They enable accurate and reliable detection of potent substances in poly-drug, authentic, case samples at trace levels that were not previously achievable.

Key features of Zeno IDA for untargeted detection of low level NSO in blood samples

- Zeno trap provides ≥90% duty cycle across the entire mass range for MS/MS acquisition
- Improved duty cycle leads to an MS/MS sensitivity increase, resulting in higher numbers of detections, improved spectral library matching and increased confidence in identification
- MS/MS sensitivity improvements of ~9X, on average, across all MS/MS fragments for the positively identified substances
- Increased MS/MS sensitivity leads to confident detection of low level NPS, metabolites and other potent drugs in discarded authentic postmortem case samples, providing the necessary evidence to support medicolegal death investigations



Experimental details

Target analytes: An NSO panel including 3 newly emerging non-fentanyl opioids (brorphine, isotonitazene, metonitazene), one metabolite (4'-hydroxy nitazene) and two halogenated fentanyl analogs (*para*-fluorofentanyl and *para*-chlorofentanyl) was selected for method development. A 1 µg/mL standard mixture containing the 6 target analytes and a 1 ng/mL fentanyl-D5 internal standard solution were prepared in water.

Calibrator preparation: The 1 μ g/mL standard mixture containing the 6 target analytes was used to fortify 500 μ L of human whole blood. This freshly spiked whole blood mixture was used to prepare a series of 9 calibrator solutions covering concentrations ranging from 10 pg/mL to 100 ng/mL.

Sample preparation: NSO were extracted from human whole blood using a liquid-liquid extraction (LLE) procedure summarized in Figure 2.

Load to tube	$\bullet 500~\mu L$ human whole blood spiked with calibrator solutions
Load to tube	•25 μL of 1 ng/μL IS stock solution
Load to tube	•1mL of Borax buffer, pH 10.4 and vortex for 5 sec
Load to tube	•3 mL of 70:30 n-butyl chloride : ethyl acetate
Rotate	•Cap and rotate for 10 min at 40%
Uncap & Freeze	•Uncap the tube and freeze at -80°C for 15 min
Transfer	Transfer supernatant to new tubes
Load to tube	•100 μL of HCl in MeOH
Dry	•Dry down in TurboVap at 35 °C, 10 psi for 30 min
Reconstitute	•Add 200 μL of 95:5 A:B to tube and vortex
Transfer	\bullet Transfer to ALS glass vial and inject 10 μL onto instrument

Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used for selectively extracting drugs from human whole blood samples for analysis with the ZenoTOF 7600 system.

Liquid chromatography: HPLC separation was performed on an ExionLC system using a Phenomenex Kinetex C18 column ($50 \times 3.0 \text{ mm}$, $2.6 \mu \text{m}$, 00B-4462-Y0). Mobile phase A (MPA) and mobile phase B (MPB) were ammonium formate (pH 5) and formic acid in methanol and acetonitrile, respectively. The flow rate was 0.4 mL/min with a total LC runtime of 15.5 minutes. The injection volume was 10 μ L.

Mass spectrometry: MS and MS/MS data were collected for each sample using Zeno IDA for optimal sensitivity on the ZenoTOF 7600 system. Data acquisition consisted of a TOF MS scan to collect accurate mass precursor ions from 100 to 700 Da, followed by a TOF MS/MS full scan ranging from 25 to 700 Da to ensure all fragments were captured for identification using a maximum of 16 candidate ions. Data was acquired using SCIEX OS software 2.0.1. **Data analysis:** Data was processed using SCIEX OS software 2.0.1. Detection and integration of the peaks from the background was accomplished using the MQ4 algorithm in the Analytics module of the software where quantitative and qualitative analyses were performed. Positive analyte identification was accomplished based on confidence criteria as previously described.² The four main confidence criteria used include mass error (M), retention time (R), isotope ratio difference (I), and library score (L). An in-house library was used to perform spectral library matching and identification of the drugs present in the discarded authentic postmortem case samples.

Optimized IDA method leads to accurate and reliable drug quantification

Information dependent acquisition (IDA) is a non-targeted data dependent acquisition technique that provides high confidence in compound identification by generating high-resolution, accurate mass spectra in both MS and MS/MS modes for spectral library matching or for structural elucidation purposes. Accurate quantification can also be performed simultaneously using the accurate mass of precursor ions from the TOF MS experiment.

A series of 9 calibrator solutions were prepared by spiking control human whole blood samples with the 6 targeted analytes at final concentrations ranging from 10 pg/mL to 100 ng/mL. The series of calibrator solutions were injected to evaluate the quantitative performance of the system and its ability to accurately measure low level analytes with a high level of precision and accuracy in TOF MS mode. Each calibrator was injected in triplicate.

Figure 3 shows representative extracted ion chromatograms (XICs) for A) metonitazene and B) isotonitazene, two highly potent NSO that have been linked to accidental drug overdoses at low concentrations. The series of XIC displays shows the resulting signal for a blank injection (left) and for concentrations ranging from 10 pg/mL (LLOQ) to 100 ng/mL for metonitazene and from 50 pg/mL (LLOQ) to 100 ng/mL for isotonitazene, respectively. Figure 3 also displays the statistical results from the peak area integration of A) metonitazene and B) isotonitazene. Excellent precision and accuracy were observed across the series of calibrators, proving the robustness of the assay. Full quantification, including detection and integration of the peaks and area, concentration and guantitative performance value calculations (precision and accuracy) was automatically performed in Analytics in SCIEX OS software. The software is designed for quick, intuitive and streamlined data processing with accurate and reliable results.

🔅 ZenoTOF 7600 system



A Metonitazene

Matrix Blan	k 10 pg/mL (LLOQ)	50 pg/mL	100 pg/mL	500 pg/mL	1 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL	100 ng/mL
Matte Bark with E. Meteoropeus (Reck. (2010)) an (2010) and(3), (ample Solar-3) Ana Nyi, Pagine Nyi, VI. Aple an	MPC (peeds 100 cqcm) - Sees (Nr. 1986, 815 See 2001 arR), Lamph Soles B. Anna (1986), Pargin 2 (Min), 47 5 59 min.	4473 (prod. 128 rg/s). [Jon 10: 10: [Jon 201] arR]; (argk lots:) Ana 1274, Ingli 1286; 17.54 na.	MECQUER KETANDER - Dere DES MANN, KERTUNGEN (UND AND, Langerbeiter I) Anne 1.0044, Hagde 1.2014, HT 518 min	MCC (product) Complete (Server (MCC Manuer, 2010) (2010) (2010), (complete (address in the Activation of the Activation	Mr1 (product a signed - Janes Die Medicale J100 Jan (2001 arM)), completions (5 Anna (2004), respire 400044, 47 5 10 mm	MPC (product 5 regime) - Jama (Nr. Mattacol, 2018 Jam 2016) ar#(), Earryte States (). Area: 400 MeV, People 3 (1984), 47: 518 mas.	Williams Highs Dec W. West, 2013a 2014R, and bits 3 for \$300, multi \$200, 01 10 years	1973 (gando 36 nghit), - Jana (16 - Milan, 2013 Jan 3010 (art9)), (arapit (nmc 3) Ana Alfibel, magint (1874, 17 3.58 na)	Millipsole (Minglet, Son (H. Men, 2023) and 2014 and 1 straphistic (
i = MirmyMMMM	i Aufuntari	and the second	1 - Martinesona			ina 100	400 100 100 100 100 100 100 100 100 100	ine interview in	

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
1	Metonitazene	0.01	3 of 3	9.875e-3	1.477e-3	14.95	98.75	9.474e-3	8.641e-3	1.151e-2
2	Metonitazene	0.05	3 of 3	5.166e-2	1.685e-3	3.26	103.33	5.079e-2	5.059e-2	5.361e-2
3	Metonitazene	0.10	3 of 3	9.053e-2	4.580e-3	5.06	90.53	8.525e-2	9.292e-2	9.342e-2
4	Metonitazene	0.50	3 of 3	5.183e-1	2.291e-2	4.42	103.66	5.432e-1	4.982e-1	5.134e-1
5	Metonitazene	1.00	3 of 3	1.023e0	1.640e-2	1.60	102.34	1.039e0	1.026e0	1.006e0
6	Metonitazene	5.00	3 of 3	5.091e0	5.074e-2	1.00	101.82	5.086e0	5.043e0	5.144e0
7	Metonitazene	10.00	3 of 3	9.814e0	3.087e-1	3.15	98.14	1.011e1	9.492e0	9.842e0
8	Metonitazene	50.00	3 of 3	5.137e1	8.919e-1	1.74	102.73	5.219e1	5.042e1	5.149e1
9	Metonitazene	100.00	3 of 3	9.870e1	2.265e0	2.29	98.70	9.996e1	1.000e2	9.608e1

B Isotonitazene

Matrix Blan	k 50 pg/mL (LLOQ)	100 pg/mL	500 pg/mL	1 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL	100 ng/mL
			With the second			Wohners report i denorti - lano de la la 2010 et la construir i servici e la construir i denorti - la construir - la cons	Windowski strani i unich i kana i Uroba i Urob	

	Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
►	1	Isotonitazene	0.01	0 of 3	N/A	N/A	N/A	N/A	1.706e-1	9.531e-1	₩/А
	2	Isotonitazene	0.05	3 of 3	5.563e-2	5.110e-3	9.19	111.27	5.854e-2	4.973e-2	5.863e-2
	3	Isotonitazene	0.10	3 of 3	9.682e-2	1.776e-3	1.83	96.82	9.478e-2	9.801e-2	9.767e-2
	4	Isotonitazene	0.50	3 of 3	5.135e-1	1.879e-2	3.66	102.70	4.994e-1	5.062e-1	5.348e-1
	5	Isotonitazene	1.00	3 of 3	9.619e-1	4.868e-2	5.06	96.19	1.011e0	9.601e-1	9.141e-1
	6	Isotonitazene	5.00	3 of 3	4.974e0	1.625e-1	3.27	99.47	5.091e0	5.042e0	4.788e0
	7	Isotonitazene	10.00	3 of 3	9.240e0	2.412e-1	2.61	92.40	9.289e0	8.977e0	9.452e0
	8	Isotonitazene	50.00	3 of 3	5.035e1	5.170e-1	1.03	100.70	5.091e1	5.025e1	4.989e1
	9	Isotonitazene	100.00	3 of 3	1.005e2	7.097e-1	0.71	100.46	1.013e2	1.002e2	9.992e1

Figure 3. Extracted ion chromatogram (XIC) traces and statistical results for A) metonitazene and B) isotonitazene, two potent NSO targeted in this study. XIC traces and resulting statistics panes from 10 pg/mL (LLOQ) to 100 ng/mL for: A) metonitazene and from 50 pg/mL (LLOQ) to 100 ng/mL for B) isotonitazene, respectively. Both NSO showed excellent accuracy and precision across the calibration levels, proving the overall robustness of the assay.

XIC area values resulting from the TOF MS experiment were used to generate regression plots for each of the 6 targeted analytes. Figure 4 shows the resulting calibration curves which demonstrate excellent linearity across the concentration ranges analyzed. They were calculated with R² values observed to be greater than 0.99 for all 6 targeted NSO.

Table 1 lists the name, the calibration range, linear correlation value (R^2), and LLOQ, as well as the accuracy and precision reported at the LLOQ for each of the 6 target analytes used in this panel. These values demonstrate the quantitative performance of the ZenoTOF 7600 system in TOF MS mode.



Figure 4. Excellent linearity for the 6 targeted NSO. Calibration curves resulting from the series of 9 calibrators extracted from human whole blood at concentrations ranging from 10 pg/mL to 100 ng/mL. R^2 values greater than 0.99 were observed for the 6 targeted analytes.



Table 1. Statistical results for the 6 targeted drugs. The table includes calibration range, linear correlation coefficient (R² Value), and LLOQ, as well as the accuracy and precision at the LLOQ for each of the 6 targeted drugs.

Compound	Calibration Range (ng/mL)	Linear Correlation (R2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
Brorphine	0.05 - 100	0.99803	0.05	88.95	8.47
Isotonitazene	0.05 - 100	0.99950	0.05	111.27	9.19
Metonitazene	0.01 - 100	0.99931	0.01	98.75	14.95
4-Hydroxy Nitazene	0.1 - 100	0.99490	0.1	103.58	7.52
para-Fluorofentanyl	0.5 - 100	0.99765	0.5	85.75	2.23
para-Chlorofentanyl	0.01 - 100	0.99712	0.01	88.24	8.17

Zeno trap technology leads to MS/MS sensitivity gains

QTOF mass spectrometers commonly make use of an orthogonal TOF geometry which has been shown to maximize MS and MS/MS resolution and mass accuracy for an entire spectrum, but results in a significant loss of ions through this region of the MS (only 5-20% duty cycle).¹ To overcome this limitation, a Zeno trap was added at the end of the collision cell on the ZenoTOF 7600 system, which increases the duty cycle in the orthogonal injection region of the MS to ≥90% across the entire mass range. Therefore, the technological enhancements on the ZenoTOF 7600 system significantly increase MS/MS sensitivity which results in improved MS/MS spectral quality at low analyte concentration. This improvement ultimately yields improved MS/MS spectral library matching which provides greater confidence in analyte identification.

Zeno MS/MS increases confident identifications of low drug levels in authentic postmortem case samples

The MS/MS sensitivity improvements resulting from the use of the Zeno trap on the ZenoTOF 7600 system was investigated by analyzing discarded authentic postmortem case samples from subjects suspected of NSO ingestion resulting in accidental overdoses. These biological specimens were prepared using the aforementioned LLE procedure. Data were acquired on the ZenoTOF 7600 system with both the Zeno trap on and off for each sample and the results were compared to assess the impact of the MS/MS sensitivity gains. The concentrations of the targeted NSO detected in the discarded authentic postmortem case samples were calculated automatically in SCIEX OS software using the calibration curves generated for each of the 6 target analytes. Each case sample was run in triplicate.

Case study 1

Figure 5 (top) shows the results table from the analysis of discarded authentic postmortem case sample #1, using Zeno IDA, where 10 analytes were successfully identified. Figure 5 (bottom) also displays the XIC, TOF MS and TOF MS/MS spectra of two representative drugs positively identified in the sample: methamphetamine and 4-(Trifluoromethyl) U-47700, a potent synthetic opioid that has been reported to cause opioidlike effects similar to heroin and fentanyl. The results table shows the successful detection of two of the targeted NSO: parachlorofentanyl and metonitazene, as well as other non-targeted NPS such as 4-(Trifluoromethyl) U-47700 and fluorofentanyl (the para and meta isomers were not resolved chromatographically). The presence of fentanyl analogs (para-chlorofentanyl and para-/meta- fluorofentanyl) and the potent synthetic opioid 4-(Trifluoromethyl) U-47700 suggest that the subject ingested a preparation originating from the illicit drug market. The presence of multiple potent NSO could support the case of combined opioid drug toxicity leading to death. Positive identification determination was accomplished using the four confidence criteria and sorted out using the traffic light system. The mass errors (ranging from -4.3 to 0.8 ppm), the mass spectra library scores (ranging from 76 to 100%) and the combined scores (ranging from 82.677 and 97.828%) provided excellent measures of the confident identification of the ten compounds in the discarded postmortem sample #1.



Discarded authentic postmortem case sample # 1



Figure 5. Results from authentic postmortem case study #1. (Top) Results table in SCIEX OS software showing the analytes positively identified in postmortem case sample #1 along with mass error, library score and combined score using the confidence criteria. (Bottom) XICs, TOF MS and TOF MS/MS spectra collected provide detailed and confident identification of two of the positively identified analytes: methamphetamine and 4-(Trifluoromethyl) U-47700.

Discarded authentic postmortem case sample # 2

Zeno trap off para-chlorofentanyl N-propylamphetamine ed Mass T Error... RT Confi... Library Library Score Sample Name Type Component Name Found At Mass ∀ Mass Error (... ⊽ Librar.. Fox Sample: Brorphine... Unknown para-chlorofenta para-Chi • 371.1880 Tor Sample: Brophine... Unknown para-Chirotentary Tor Sample: Brophine... Unknown Tor Sample: Brophine... Unknown Nethample: Brophine... Unknown Sample: Brophine... Unknown Car Sample: Brophine... Unknown Tor Sample: Brophine... Unknown Tor Sample: Brophine... Unknown Septimie: Stample: Stampline... Unknown Feataryl N/A 150.1271 -4.3 95.3 • N/A N/A 178.1582 -4.9 N-Propy 20.9 67.2 99.4 31.5 100.0 94.3 92.5 180.1742 -2.6 195.0874 286.1433 Caffeine -1.4 -1.7 -0.9 × × × × × × × Morphin Fentanyl × 7.2271 Tox Sample: Brorphine... Unknown Clon N/A 354.0741 -1.5 Clonazo Tox Sample: Brorphine... Unknown ortho-Fluorof N/A N/A 355.2174 -17 ortho-Flu Name CAS# Formula MM (Da) Fit Rev.Fit Purity CE (eV) Name CASE Formula MM (Da) Fit Rev. Fit Purity CE (eV) 89.3 49.4 88.0 Tox Sample: Brorphine... Unknown meta-Fluorofentany 355.2174 -1.7 meta-Flu Tox Sample: Brorphine... Unknown Halop Tox Sample: Brorphine... Unknown Brorph Tox Sample: Brorphine... Unknown Verap ridol • 376.1466 -2.0 Zeno trap off Zeno trap off ~10x Zeno Zeno trap on Gain Calculated V Error Confi... Sotope Library Confi ... Found At Mass ▼ Mass Error (.... ▼ Librar... Library Score ira-Chl para-o 0.1275 Tox Sample: Brorphine... Unknown Aetham Tox Sample: Brorphine... Unknown N-Propylamphetamin 178.1586 -2.3 N-Propyl 100 Tox Sample: Brorphine... Unknown Memanti Tox Sample: Brorphine... Unknown Caffeine N/A 180.1746 -0.4 Memanti 97.8 N/A 195.0876 -0.1 Caffeine 99.4 Tax Sample: Brophine... Unknown Caffeine Tax Sample: Brophine... Unknown Morphine Tax Sample: Brophine... Unknown Fentanyl Tax Sample: Brophine... Unknown cflonzolam Tax Sample: Brophine... Unknown ortho-Fluoro N/A N/A N/A N/A 86.1435 78.6 Fentanyl Clonazol. ortho-Flu meta-Flu. 37.2274 100.0 -0.1 -1.5 -0.9 -0.9 -1.8 54.0747 355.2177 8 Lorary search Results Name CAS# Formula MM (Da) Fit Rev. Fit Purity CE (eV) Name CAS# Formula MM (Da) Fit Rev. Fit Purity CE (e Tox Sample: Brorphine... Unknown meta-Fluorofentary 55.2171 Haloperi Tox Sample: Brorphine... Unknown Haloperidol Tox Sample: Brorphine... Unknown Brorphine N/A 76.1467 93.0 7.611e-1 00.1010 -2.3 Brorphin 4.00 Zeno trap on Zeno trap on 1005 331

Figure 6. Results comparison between Zeno trap on and off for authentic postmortem case study #2. (Top) Results table and representative TOF MS/MS spectra with (top) and without (bottom) the Zeno trap enabled. The use of the Zeno trap resulted in a 10x improvement, on average, in sensitivity, which resulted in greater confidence in analyte identification confirmation through MS/MS spectral library matching. р5



Case study 2

The use of the Zeno trap for this qualitative workflow should provide substantial improvements in the observed TOF MS/MS spectral quality which should ultimately result in greater confidence in spectral library matching confirmation. Figure 6 (left) shows the results table from the analysis of discarded authentic postmortem case sample # 2 without (top) and with (bottom) activation of the Zeno trap. The analysis of this sample with the Zeno trap on resulted in greater library confidence for the majority of the positively identified compounds, as evidenced by comparing the green icons (bottom table) with the Zeno trap on to the red and yellow icons (top table) with the Zeno trap off.

For example, the library score for the identification of Ichlorofentanyl and N-propylamphetamine (ISTD) increased from 20.8% to 86.1% and from 20.9% to 99.4%, respectively, when the Zeno trap was activated. This drastic improvement in library score is the consequence of the MS/MS sensitivity enhancements afforded by the Zeno trap, which resulted in improved TOF MS/MS spectral quality. The sensitivity gains are shown in the TOF MS/MS spectra comparison for *para*chlorofentanyl and N-propylamphetamine in Figure 6 (right). Overall, the average library score for the positively identified analytes in this sample increased from 74.5% to 94.4% when the Zeno trap was activated. It also resulted in a 10x improvement, on average, in sensitivity, which resulted in greater confidence in analyte identification confirmation through MS/MS spectral library matching.

Discarded authentic postmortem case sample # 3

Zeno trap off

ample Name 🛛 🗸	Sample 🚽	Component Name	C.	alculated 🚽	Mass Error	RT Confi	Isotope Confi	Library Confi	Found	Mass .	7 Librar 5	Library V	Spectrum from 022820	121 Tax Sample - Isoto99	min Precursor: 180.2 D	+1, CE: 35.0	Spectrum from 0	1282021 Tox Sample - Iso	oto52 min Precursor	r: 381.3 Da, +2, CE: 35.0
Samperane 1	Туре .	component tunic i	Cor	ncentrat					At Mass	Error (Score	35				1			391.4307
x Sample: Isotonitaz	Unknown	para-chlorofentanyl	< 0		~	~	~	٠	371.1880	-1.2	para-Chl	22.8	30				30 -			
x Sample: Isotonitaz	Unknown	Phentermine	N/4	A.	~	~	~	~	150.1270	-4.7	Phenter	75.7	8 2				C cbi			
x Sample: Isotonitaz	Unknown	N-Propylamphetamine	N/4	A	~	~	~		178.1580	-4.5	N-Propyl	38.7	20 -				Q100 20 -			
x Sample: Isotonitaz	Unknown	Memantine	N/4	A	~	~	~	٠	180.1740	-3.9	No Match	0.0	A D				2			
ix Sample: Isotonitaz	Unknown	Caffeine	N/4	A.	~	~	~	~	195.0878	0.6	Caffeine	99.2	10				10 -			
n Sample: Isotonitaz	Unknown	Morphine	N//	A	~	~	~	٠	286.1433	-1.7	Morphine	17.5	.1							
ox Sample: Isotonitaz	Unknown	Fentanyl	N/4	A	~	~	~	~	337.2276	0.4	Fentanyl	100.0		50 100	150			50 100 150	200 250	300 350 400
ox Sample: Isotonitaz	Unknown	ortho-Fluorofentanyl	N/4	A	~	~	~	~	355.2177	-0.8	ortho-Flu	92.5	Library Search Resul	Mass.	/Charge, Da	G	 Library Search 	Results	mess/unerge, Da	6
ox Sample: Isotonitaz	Unknown	meta-Fluorofentanyl	N/4	A.	~	~	~	~	355.2177	-0.8	meta-Flu	89.3	Name CAS#	Formula MM (Da)	Fit Rev. Fit Pur	ity CE (eV)	Name C	AS# Formula MM	(Da) Fit Rev. F	Fit Purity CE (eV)
ox Sample: Isotonitaz	Unknown	5-Aminoisotonitazene	N//	A	~	~		٠	381.2651	0.5	No Match	0.0								
ox Sample: Isotonitaz	Unknown	Isotonitazene	3.45	99e0	~	~	~	~	411.2383	-1.9	Isotonita	83.2								
Leno tra	p or	1																		
Sample Name 3	Sample	7 Component Name 🔻		Calculated	Mass ⊽ Error.	RT Confi	Isotoj Confi	e Libr Con	ary Found At Mas	s ♥ Mass Error	v Librar.	Library Score	Spectrum from 02282	2021 Tox Sample - Isot14	amin Precursor: 188.2 D	. +1, CE: 35.0 140.1737	Spectrum from I	12282021 Tox Sample - Is	10to25 min Precursor	rr: 381.3 Da, +2, C8: 35.8 381.3157
Sample Name	Sample Type	7 Component Name T		Calculated Concentra	▼ Mass Error.	. RT Confi	Isotoj Confi	e Libr Con	ary fi At Mas	s ♥ Mass Error I	Librar.	Ubrary Score	Spectrum from 02282	2021 Tox Sample - Isot14	1 min Precursor: 188.2 D	6, +1, CE: 35.0 100.1737	Spectrum from	12282021 Tox Sample - Is	100025 min Precursor	er: 381.3 Da, +2, CE: 35.8 381.2157
Sample Name 3	Sample Type Unknown	Component Name para-chlorofentanyl Departermine		Calculated Concentra < 0	▼ Error.	. RT Confi	Isotoj Confi	e Libr Con	ary fi Found At Mas 371.1881	s ▼ Mass Error 1 -1.0	j▼ Librar. para-Ci	- V Library Score 7	Spectrum from 02282 400-	2021 Tox Sample - Isot14	i min Precurson: 188.2 D	5, +1, CE: 35.0 180. 1 737	Spectrum from I	2282021 Tox Sample - Is 58,0510	oto25 min Precursor	in 301.3 Da, +2, CE: 35.0 301.2157 301.2608
Sample Name To ox Sample: Isotonitaz ox Sample: Isotonitaz ox Sample: Isotonitaz	Sample Type Unknown Unknown	7 Component Name Y para-chlorofentanyl Phentermine Naftorndamphetamine		Calculated Concentra < 0 N/A N/A	▼ Mass Error.	RT Confi	Isotoj Confi	e Libr Con	ary film Found At Mas 371.1881 150.1273 118.1565 118.1565	s ▼ Mass Error 1 1 -1.0 1 -2.6	i ↓ ↓ Librar. para-Cl Phenter	Library Score 7	Spectrum from 02282 400 - 55 - 310 -	2021 Tox Sample - Isot14	i min Precurson: 188.2 D 198	s, +1, CE: 35.0 380.1737	Spectrum from 1 200 - 3 150 -	2282021 Tox Sample - Is 59.0510 46.0437	iote25 min Precursos	in 381,3 0a, +2, CE: 35,8 381,2557 381,2608
Sample Name T ox Sample: Liotonitaz ox Sample: Liotonitaz ox Sample: Liotonitaz	Sample Type Unknown Unknown Unknown	7 Component Name para-chlorofentary/ Phentermine N-Propylamphetamine Meroanine		Calculated Concentra < 0 N/A N/A N/A	▼ Error.	RT Confi	Isotoj Confi	e Libr Con	ary fi Found At Mas ' 371.1881 ' 150.1273 ' 178.1585 ' 180.1248	s ▼ Mass Error 1 -1.0 -2.6 -3.2 -4.0	i ↓ Librar. para-Cl Phente N-Prop	. ▼ Library ▼ Score ▼ II 83.6 97.4 VI 99.3 N 93.1	Spectrum from 02202 400 - 75 - 310 - 26 210 -	2021 Tox Sample - Isot14 57.0681	1 min Precurson: 188.2 D 188 189	s, +1, CE: 35.0 100.1737 1381 1350	Spectrum from	2282021 Tox Sample - Is 55.0510 46.0637 57.0326	ota25 min Precursor	in 381.3 Da, +2, CE: 35.8 381.2557 381.2608 381.2031
Sample Name v xx Sample: Isotonitaz xx Sample: Isotonitaz xx Sample: Isotonitaz xx Sample: Isotonitaz xx Sample: Isotonitaz	Sample Type Unknown Unknown Unknown Unknown	Component Name para-chlorofentanyl Phentermine N-Propylamphetamine Merrantine Cottrine		Calculated Concentra < 0 N/A N/A N/A N/A	▼ Mass Error.	RT Confi	Isotoj Confi V V	Libr Con	ary fi Found At Mas 7 371.1881 7 150.1273 7 178.1585 7 180.1740 7 150.8880	s ▼ Mass Error 1 -1.0 -2.6 i -3.2 -4.0 2.0	i ▼ Librar. para-Cl Phente N-Prop Memar	. ▼ Library ▼ Score ▼ II 83.6 97.4 93.1 93.1 93.4	Spectrum from 0232 410 - 53 300 - 25 20 - 20 - 20 - 20 - 20 - 20 - 20 - 20 -	57.0681	198.2 D 198.2 D 198 126.8777 180 2.848 J	n, +1, CE: 35.0 100.1737 1381 1558	Spectrum from	2282021 Tox Sample - Is 556/510 46.9637 57.8326 131.6949	oto	er 361.3 Du, +2, CE: 15.8 361.257 361.2008 361.2011 8.2104 381.2036
Sample Name V x Sample: Isotonitaz x Sample: Isotonitaz x Sample: Isotonitaz x Sample: Isotonitaz x Sample: Isotonitaz x Sample: Isotonitaz x Sample: Isotonitaz	Sample Type Unknown Unknown Unknown Unknown Unknown	Component Name para-chlorofentanyl Phentermine NePropylamphetamine Mernantine Caffeine Morrahine		Calculated Concentra < 0 N/A N/A N/A N/A N/A N/A N/A	▼ Mass Error. ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓	RT Confi	Isotoj Confi V V V	Libr Con	ary fi 7 371.1881 7 150.1273 7 178.1585 7 180.1740 7 195.0880 7 296.1433	Mass Error 1 1.0 2.6 3.2 4.0 1.7	Librar. para-Cl Phenter N-Prop Memar Caffein	□ □ Library Score □ sl 83.6 97.4 yl 99.3 sti 99.3 sti 99.3 sti 99.3 sti 99.1 sti 99.4	Spectrum from 0202 400 - 10 - 200 - 210 - 210 -	57,9441	100 Precursor: 108.2 D 100 120.8777 100 120.8777 100 100 100 100 100 100 100 100 100	, +1, CE: 35.0 100.1737 1301 1550 1625	Spectrum from	2282021 Tox Sample - Is 550/510 46.0637 57.0326 131.0040	uoto	n: 381.3 Du, +2, CE: 35.8 381.2157 381.2001 381.2001 81.2304 41.2304
Sample Name S Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz	Sample Type Type Type Type Type Type Type Typ	Component Name T para-chlorofentanyl Phenternine N-ProgNamphetamine Memantine Caffeine Morphine Eestand		Calculated Concentra < 0 N/A N/A N/A N/A N/A N/A N/A	▼ Mass Error.	RT Confi	Isotoj Confi V V V V	e Libr Con	ary fi Found At Mas 371.1881 371.1881 150.1273 178.1585 180.1740 195.0800 195.0804 195.08143 331.2212 331.2212	Mass reference i -1.0 -2.6 -3.2 -4.0 2.0 -1.7 -0.7	and para-Cl para-Cl Phenter N-Prop Memar Caffein Morphi Eentam	■ ▼ Library Score ▼ sl 83.6 • • yl 99.3 • • sti 93.1 • • e 99.4 • • • ne 79.7 4 • • •	Spectrum from 02212 410 - 15 - 210 - 210 - 0	57.0641 79.0536 2021 Tox Sample - Loct14	1min Precurson 188.2 D 198 100 126.8777 100 126.8649 120.8649 120.8649 120 120 120 120 120 120	s, +1, CE: 35.0 100.1737 1101. 1550 1625 331	Spectrum from 0 200 100 200 200 200 200 200 200 200 20	2282021 Tox Sample - Is 550/510 46.9637 77.9226 131.0849 50 100 150	000	m 3613 0.4, +2, CE: 55.8 3612157 3612031 42364 19122334 360 250 400
Sample Name Sample Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz Sample: Isotonitaz	Sample S Sample S Unknown Unknown Unknown Unknown Unknown Unknown	Component Name para-chlorofentanyi para-chlorofentanyi Phentermine Ne-Programphetamine Mernantine Caffeine Morphine Fentanyi enta-chlorofentanyi		Calculated Concentra <0 N/A N/A N/A N/A N/A N/A N/A N/A	▼ Mass Error.	RT Confi	Boto Confi V V V V V V V V	Con	ary file Found At Mass 371.1881 1 150.1273 1 150.1273 1 150.1273 1 180.1740 1 195.0880 2 286.1433 3 283.2272 4 4 353.2172	Mass - 1.0 - 2.6 - 3.2 - 4.0 2.0 - 1.7 - 0.7 - 0.7 - 0.7 - 0.7	Librar. para-Cl Phente N-Prop Mermar Caffein Morphi	V Library Score S	Spectrum from 82282 410 13 330 20 20 40 10 0	57.0681 59.0536 100 50 Mai	198 199 190 190 190 190 190 190 190 190 190	s, +1, CE: 35.0 100.1737 1101.1737 1101.1737 1101.1737 1625 3331	Spectrum from 0 200 15 - X 200 100 100 100 0	2282021 Tox Sample - Is 55.0510 57.0326 131.0849 50 100 150	Jan Stand St	1912 Du, +2, CE: 35.8 3912 203 3912031 3912031 3912031 3912031 3912031 3912031 3912031
Sample Name S Sample Isotonita Sample Isotonita Sample Isotonita Sample Isotonita Sample Isotonita Sample Isotonita Sample Isotonita Sample Isotonita Sample Isotonita	Sample S Sample S Unknown Unknown Unknown Unknown Unknown Unknown	Component Name para-chlorofentanyi Phentemine N-Propylamphetamine Mernartine Coffeine Morphine Fentanyi entanyi entanyi entanyi		Calculated Concentra < 0 N/A N/A N/A N/A N/A N/A N/A N/A N/A N/A	▼ Mass Error.	RT Confi	Sotoq Confi V V V V V V V V V V V V V V V	e Libr Con	ary III Found At Mass 7 371.1881 7 150.1273 7 178.1585 7 180.1740 7 195.0880 7 375.2727 7 355.2177 7 355.2177	Mass 1.0 2.6 3.2 4.0 - 2.0 1.7 - 0.7 - 1.0 - 1.0 - 2.6 - 3.2 3.2 3.2 3.2 1.0 - 2.6 - 3.2 1.0 - 2.6 - 3.2 1.0 - 2.6 - 3.2 1.0 1.0 2.6 3.2 1.0 2.6 3.2 1.0 2.6 3.2 1.0 2.6 3.2 1.0 2.6 3.2 1.0 2.6 3.2 1.0 2.6 3.2 1.0 1.0 2.6 3.2 1.0 1.0 2.6 1.0 2.6 1.0 2.6 1.0 2.6 1.0 2.6 1.0 2.6 1.0 1.7 1.7 1.7 1.7 1.7 1.7 1.7 1.7 1.7 1.7 1.7 1.7 1.0 1.7 1.0 1	in V Librar. para-Cl Phenter N-Prop Merman Caffein Morphi Fentany ortho-F	- V <mark>Library V Score V</mark> al 83.6 97.4 yl 99.3 ti 93.1 te 99.4 ne 79.7 4 100.0 fu 83.1 y 72.3	Spectrum fram 10221 480 - 10 - 300 200 - 100 8	57,9441 57,9441 59 50 50 50 50 50 50 50 50 50 50 50 50 50	188 2 D 188 2	s, +1, CE: 35.0 106.1737 1359 1625 3331	Spectrum from 1 200 - 15 - 150 - 100 - 100 - 0 -	2282021 Tex Sample - Is 55.0510 56.0507 57.0226 101.0840 50 100 350 Results	200 259 Mass/Change, De	er 341.3 Da, +2, CE 35.8 341.2537 341.2689 341.2031 44.2144, 341.2034 340 350 400
Sample Name 3 Sample Isotonitaz Sample Isotonitaz	Sample Type Unknown Unknown Unknown Unknown Unknown Unknown Unknown Unknown	Component Name pan-chordentanyl Phentemine N-ProgNamphetamine Mernantine Coffeine Morphine Fentanyl resta-Fluorofentanyl resta-Fluorofentanyl		Calculated Concentra < 0 N/A N/A N/A N/A N/A N/A N/A N/A N/A N/A	▼ Mass Error. ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓	RT Confi	Isotoj Confi V V V V V V V V V V V	Libr Con	ary Inn Found At Mass 7 371.1881 7 150.1273 7 178.1585 7 180.1740 7 195.0880 7 337.2272 7 355.2177 7 355.2177 7 355.2177	Mass Terror 1 - 1.0 - 2.6 - 3.2 - 4.0 - 4.0 - 2.0 - 1.7 - 0.7 - 1.0 - 1.0 - 1.0 - 1.7 - 1.0 - 1.7 - 1.0 - 1.7 - 1.0 - 1.0 - 1.0 - 3.2 - 1.7 - 1.0 - 1.0 - 1.0 - 1.7 - 1.0 - 1.0 - 1.0 - 1.0 - 1.7 - 1.0 - 1.0 - 1.0 - 1.0 - 1.0 - 1.0 - 1.7 - 1.0 - 1.0	Librar. para-Cl Phente N-Prop Memara Caffein Korphi Fentany ortho-F meta-F	Library Library Core Mail 83.6 7 Mail 97.4 90.3 Mail 99.3 9 Mail 99.4 9 Mail 100.0 10 Mail 10.0 10 Mail 77.3 10	Spectrum fram 12202 400 30 200 200 8 200 8 100 8	57.0441 78.0536 100 59 100 59 100 78.0536 100 59 100 Mas Mas	100 100 100 100 100 100 100 100 100 100	5, +1, CE, 35,0 116,1737 13061 13580 13101 131	Spectrum from 200 5 5 50 0 0 2 8 50 0 0 2 8 50 0 0 2 8 50 0 2 0 2 8 50 50 50 50 50 50 50 50 50 50 50 50 50	2222021 Tox Sample - Is 59.8510 59.8510 50.310 50.5000 50.5000 50.5000 50.5000 50.500	200 250 Musi/Change, Da (Da) Fit Rev. 1	in 381.3 Du, +2, CE: 35.8 381.2157 381.2003 381.2003 44.2144 310 350 400 TR Punity CE (cef)

memantine

Zeno trap on

Figure 7. Results comparison between Zeno trap on and off for authentic postmortem case study #3. (Top) Results table and representative TOF MS/MS spectra with (top) and without (bottom) the Zeno trap enabled. The use of the Zeno trap enabled acquisition of a much richer MS/MS spectra that contained unique fragment ions that were used for confident compound identification.

Case study 3

Figure 7 shows the results tables and representative TOF MS/MS spectra comparison from the analysis of discarded authentic postmortem case sample #3. Similar observations can be drawn from the observations made for the analysis of the first case sample. First, the use of the Zeno trap resulted in the confident detection of all ten compounds with high confidence as evidenced by the high library scores ranging from 77.3 to 99.3%. Without the Zeno trap activated, analysis of this case sample resulted in two poorly matched analytes (para-chlorofentanyl and morphine returned a yellow and red library match icon, respectively) and three unmatched analytes (Npropylamphetamine, memantine and 5-aminoisonitazene returned a red library match icon) because of the poor quality of the triggered TOF MS/MS spectra. This is evidenced by comparing the TOF MS/MS spectra for two of these analytes, memantine and 5-aminoisonitazene (right). Without the Zeno trap activated, the generated TOF MS/MS spectra did not contain unique fragment ions to yield a library match. Overall, a 9x improvement, on average, in sensitivity was observed across the TOF MS/MS spectra positively identified in the three authentic postmortem case samples analyzed when the Zeno trap was activated (Figure 1). In addition, the use of the Zeno trap enabled acquisition of a much richer MS/MS spectrum that was used for confident compound identification, which resulted in an average library score increase from 56.3% to 88.6%.

5-aminoisotonitazene

Zeno trap on



A few observations can be drawn from the results highlighted in Figure 7. First, the added MS/MS sensitivity afforded by use of the Zeno trap enabled the accurate identification of 5aminoisonitazene, one of metabolites of the potent NSO isotonitazene, with a library score of 81.8%. Second, the detection of fentanyl and other fentanyl analogs (*para*chlorofentanyl and *para-/meta*-fluorofentanyl) suggest that the drug ingested by the subject might have originated from the illicit market. Although the presence of fentanyl might have been a contributing factor to the accidental overdose, the presence of the potent NSO isotonitazene and its metabolite could support the case of combined opioid drug toxicity leading to death.

Conclusions

A comprehensive and highly sensitive method for the screening and identification of potent NSO in human whole blood is described. The significant gains in MS/MS sensitivity on the ZenoTOF 7600 system yielded an improvement in confident identifications of low-level analytes through spectral library matching. The observed sensitivity gains afforded by the use of the Zeno trap resulted in a 9x improvement, on average, in TOF MS/MS sensitivity across the drugs positively identified in the authentic case samples analyzed. This improvement enabled confident identification of key drugs and metabolites at trace levels that were not previously achievable.

The MS/MS sensitivity levels afforded by ZenoTOF 7600 system provide a means to monitor low levels of ultra-potent NSO in poly-drug intake scenarios. This advancement could support the case of combined opioid drug toxicity leading to death, which offers a valuable insight into the causation of accidental overdoses.

References

- 1. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.
- vMethod Application Single-Injection Screening of 664 Forensic Toxicology Compounds on a SCIEX X500R QTOF System.

The SCIEX clinical diagnostic portfolio is For In Vitro Diagnostic Use. Rx Only. Product(s) not available in all countries. For information on availability, please contact your local sales representative or refer to www.sciex.com/diagnostics. All other products are For Research Use Only. Not for use in Diagnostic Procedures.

Trademarks and/or registered trademarks mentioned herein, including associated logos, are the property of AB Sciex Pte. Ltd. or their respective owners in the United States and/or certain other countries (see www.sciex.com/trademarks).

© 2021 DH Tech. Dev. Pte. Ltd. RUO-MKT-02-13303-B.



Headquarters 500 Old Connecticut Path | Framingham, MA 01701 USA Phone 508-383-7700 sciex.com International Sales For our office locations please call the division headquarters or refer to our website at sciex.com/offices