Technical note | 002551

Toxicology

Rapid postmortem analysis of novel psychoactive substances using a high-resolution Orbitrap mass spectrometry method

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Keywords

Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer, Vanquish Flex UHPLC system, TraceFinder software, blood analysis, drug testing, postmortem toxicology, forensic toxicology, opioids, fentanyl, bromazolam, illicit drug toxicity deaths, harm reduction, public health, toxicology surveillance, bio-surveillance, retrospective data analysis, novel psychoactive substance (NPS), targeted screening and quantification

Goal

To develop a reliable, high-throughput quantitative liquid chromatography-high resolution accurate mass spectrometry (LC-HRAM-MS) based method for postmortem drug testing and identify novel psychoactive substances (NPS) through retrospective data analysis using the Thermo Scientific™ Vanquish™ Flex UHPLC system and Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer.

Application benefits

- Expedited testing protocol for quantitative postmortem blood analysis increases laboratory productivity
- Added bandwidth to conduct valuable retrospective analysis for the identification of unknown compounds

Introduction

Illicit drug toxicity deaths deaths in Canada continue to rise and, much like the well-documented experience of the United States, many of these deaths can be attributed to opioids, including fentanyl, fentanyl analogs, and other opioid receptor agonists. The rise in drug-related deaths and the devastating effects of the opioid crisis highlight the need for increasingly sensitive toxicologic testing as well as ongoing communication between forensic researchers and public health authorities on new, potentially lethal psychoactive substances in circulation.

The B.C. Provincial Toxicology Centre (PTC) conducts postmortem drug testing for the B.C. Coroner's Service. Postmortem samples are analyzed at PTC using a routine blood drug screening procedure powered by LC-HRAM. The 12.5-minute routine run targets more than 20 unique drug compounds to determine whether potentially lethal concentrations are present. Results are then delivered within 24 hours to the coroner's office. The results are then provided in near-real-time to public health authorities and published in the coroner's regular Illicit Drug Toxicity report, which is compiled to better

understand the types of drugs being detected in recent illicit drug toxicity deaths in B.C. and to inform other agencies' public safety strategies in a timely manner. This optimized drug testing method was validated by PTC's team and significantly increased the productivity of the laboratory.

Given the challenging nature of postmortem blood samples, a robust and highly sensitive instrument is required for accurate analysis. PTC's analysis was carried out using the Q Exactive HF Orbitrap LC-MS/MS system (Figure 1) coupled with the Vanquish Flex UHPLC system (Figure 2). The mass spectrometer features an ultra-high-field Orbitrap analyzer for faster scan speed and high resolution, enabling more sample runs and precise quantification for a wide array of compound classes, while the Vanquish Flex UHPLC enables high quality separations and is easy to use.

Importantly, the output of PTC's routine blood drug screens includes robust data files from which additional value can be extrapolated. The availability of these data files combined with the drug testing productivity gains enabled by Thermo Fisher Scientific's instruments allowed PTC's scientists to also conduct retrospective data analysis that is critical to identifying unknown compounds in circulation that may pose a public health threat.

Figure 1. Q Exactive HF Orbitrap LC-MS/MS system

Figure 2. Vanquish Flex UHPLC system

Typical blood screens applied to postmortem samples for routine analysis are often not sensitive enough to detect all the drugs present in a sample. Consider the example of fentanyl, a well-known drug with robust synthetic routes. Small changes, like adding a methyl group or replacing a carbon with a nitrogen or oxygen, can lead to over 2,000 analogs, resulting in an enormous number of new potential fentanyl analogs with unknown potencies. Toxicology laboratories struggle to detect and confirm new NPS before new ones are introduced. It is estimated that about thirty to fifty new NPS are introduced each year, making it difficult for researchers to identify them, and then warn officials of especially toxic compounds in a timely manner for public health surveillance.[1]

With retrospective analysis, or re-interrogation of already acquired data with a new set of parameters, however, illicit drugs not included in the routine drug panel could be detected and presumptively identified by PTC. Using an in-house NPS data processing method, the PTC team produced data on the detection of bromazolam, a designer benzodiazepine drug that was first detected in British Columbia in January 2021, often in combination with fentanyl. It is believed to be the first report to provide concentrations of bromazolam in postmortem blood samples in Canada.[2]

Experimental

Screening method for bromazolam in postmortem blood samples

Postmortem blood samples obtained from cases of suspected illicit drug toxicity between July 14, 2020, and December 31, 2021, were screened by PTC using LC-HRAM-MS as part of routine postmortem screening. The resulting data files were then retrospectively analyzed for the presence of more than 83 drugs including bromazolam (Figure 3).

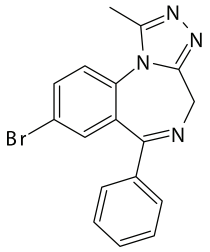

Figure 3. Bromazolam structure

Blood samples were extracted by salt-assisted liquid–liquid extraction with ice-cold methyl *tert*-butyl ether/acetonitrile (1:9, v:v) mixture. After evaporation of the organic phase, the extracts were reconstituted in methanol: Type I water (1:1, v:v) before being injected (2 µL) on a Vanquish Flex Binary UHPLC system coupled to a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer. Chromatographic separation was achieved using a Thermo Scientific™ Accucore™ Phenyl-Hexyl column (2.1 × 100 mm, 2.6 Å) using a gradient elution. Mobile phase A was 2 mM ammonium formate with 0.1% formic acid in Type I water. Mobile phase B was 2 mM ammonium formate with 0.1% formic acid in a 1:1 (v:v) mixture of acetonitrile and methanol. The flowrate was 0.5 mL/min. The total run time was 12.5 minutes. The column and the autosampler temperatures were set at 40°C and 10°C, respectively. Full scan with targeted data-dependent MS^2 was performed in the positive electrospray ionization mode with an inclusion list containing over 200 drugs, including bromazolam. The sheath gas flowrate and the auxiliary gas flowrate were set at 60 and 20 au, respectively. The spray voltage was set at 3,000 V. The capillary and the auxiliary gas heater temperatures were set at 380°C and 375°C, respectively. The S-lens RF was set to 60 V.[2]

Data processing method

The data processing was conducted using Thermo Scientific™ TraceFinder™ 4.0 software. A new psychoactive drug database was created in-house by entering the chemical formula and product ion information for bromazolam and other NPS. Bromazolam's chemical formula is $C_{17}H_{13}BrN_4$ with a protonated accurate mass of 353.03964 mass units. The main protonated fragments of bromazolam are at 325.0169, 274.1178, 205.0750, 171.0758, and 143.0693 amu, respectively. Positive results were filtered with an acceptable mass error of <10 ppm for precursor and product ions and a retention time of 6.30 min ± 10%. All positive results were reviewed for peak shape, isotopic profile, retention time, fragments and peak area response before the samples were approved for re-extraction, confirmation and quantitation by standard addition on a LC–MS/MS system.[2]

Quantitation of bromazolam using standard addition

The main advantage of standard addition is the use of a non-blank matrix (the case sample itself) for quantitation of the target compound. The case sample is used to prepare calibrators, from which the calibration curve is derived, by up-spiking the target compound at different concentrations into aliquots of the case sample. The validation parameters assessed were linearity, limit of detection (LOD), recovery, matrix effects, carryover from analyte and internal standard, and interferences from commonly encountered drugs, including but not limited to opioids, over the counter (OTC) medications, benzodiazepines, stimulants, Z-drugs, anti-depressants, anti-psychotics (N = 177).[2]

Recovery was assessed by comparing the bromazolam/alprazolam-d_5 peak area ratio from the extraction of blank blood spiked with bromazolam at a concentration of 5 and 25 ng/mL before and after extraction. Matrix effects were assessed at 5 and 25 ng/mL by comparing the bromazolam peak post-extraction and in methanol: water (1:1, v:v). Carryover was assessed by injecting a solvent blank after the calibrators. Criteria for acceptable performance were: (i) a signal-to-noise ratio of 3 at the LOD, (ii) a correlation coefficient of ~0.99, (iii) for carryover, no peak observed at the analyte or internal standard retention time, and (iv) quality control samples' accuracy within 25% of the target concentration.[2]

Results and discussion

There were 41 cases where bromazolam was detected in 2021. All cases had information about sex and 40 cases had information about age. The mean age was 42.5 (±14.0) years, and the median was 43 years. The age range observed was from 17 to 74 years. Men were predominant (80%).[2]

The standard addition protocol was validated and applied to all samples that presumptively identified bromazolam by the retrospective data analysis performed on postmortem samples. Linearity of the assay was evaluated by back calculating the calibrators' concentrations using a linear regression fit with no weighting ($r^2 > 0.99$) for each case sample. The calibration curve equation was used to calculate the concentration of bromazolam in the postmortem samples.[2]

Out of the 41 cases, seven did not have sufficient sample volume for quantitation. Twenty-seven were confirmed positive above the method's lower limit of quantitation (LOQ, 0.5 ng/mL). The remaining seven cases showed bromazolam concentrations between the LOD (0.1 ng/mL) and the LOQ.[2]

The mean bromazolam concentration observed in the cases above the LOQ was 11.4 ± 53.7 ng/mL (median concentration: 1.6 ng/mL), with a range from 0.5 to 319.3 ng/mL. Bromazolam was detected across the province, with the highest number of cases in Vancouver and Victoria.[2]

In the 41 samples where bromazolam was detected, all contained at least one additional drug. A total of 47 different drugs and/or metabolites were observed across the 41 cases, with the most prevalent being fentanyl (88% of cases) and norfentanyl, followed by methamphetamine and amphetamine (56% of cases) (Figure 4).[2]

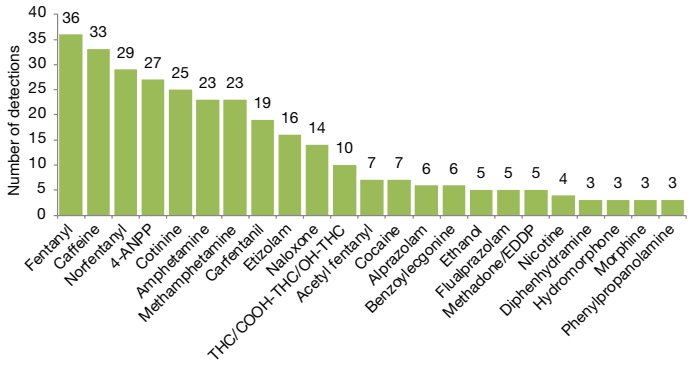

Figure 4. Co-detected drugs with bromazolam from 41 post-mortem cases

A total of 2,865 detections were observed by PTC between 2020 and 2022 (Table 1). The majority of the postmortem blood samples analyzed contained benzodiazepines (54%) and fentanyl analogs (other than fentanyl, 9.7%).

Table 1. Most common NPSs observed 2020-2022

NPS	2020	2021	2022	Total # of detections	Percentage of samples analyzed
Benzodiazepines*	20	1214	1133	2367	54%
Nitazenes	0	40	17	57	1.3%
Fentanyl analogs	4	157	262	423	9.7%
Other**	3	8	7	18	0.4%
Total	27	1419	1419	2865	

* Only designer benzodiazepines
** includes synthetic cannabinoids, stimulants, hallucinogens

Figure 5 shows the most common NPS observed during the three year period. The benzodiazepines etizolam and flualprazolam dominated in 2021. Flubromazepam was also detected, starting January 2021 but never reached the same level of etizolam and flualprazolam. Bromazolam was first detected in January 2021 but became prevalent, overtaking etizolam and flualprazolam detections in late 2022. Bromazolam is now the most detected benzodiazepine in British Columbia in postmortem blood samples. Para-fluorofentanyl is the major opioid detected other than fentanyl itself in British Columbia, whereas the carfentanil cases trend is a constant. Nitazenes were observed in the postmortem samples but rarely.

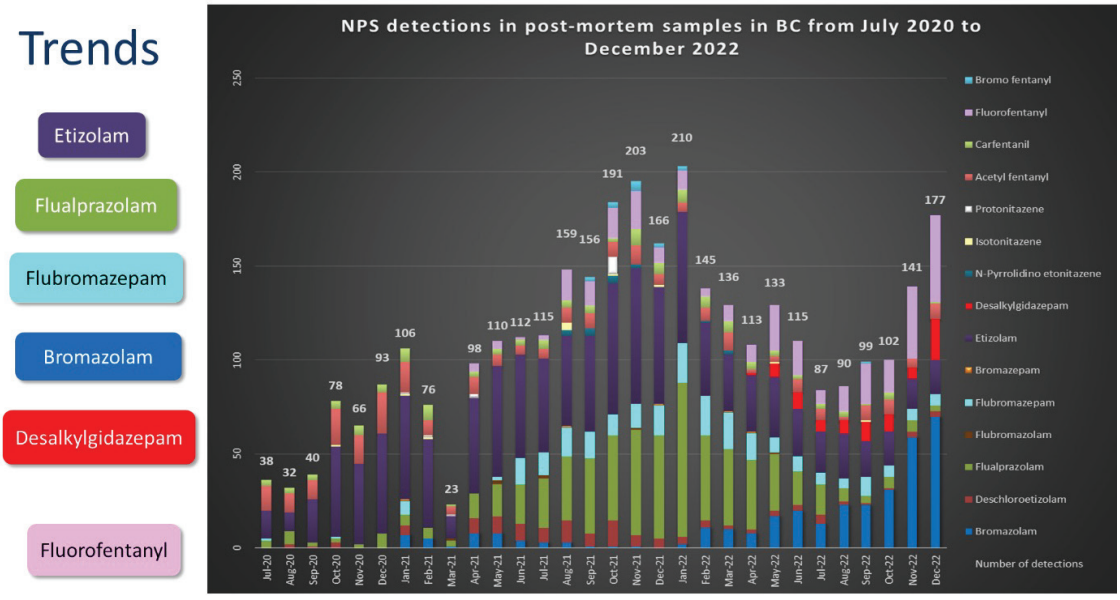

Figure 5. Trends in most common NPSs observed

Conclusions

This technical note demonstrates an LC-HRAM-MS method to perform routine blood drug screening. The drug bromazolam is a prevalent designer benzodiazepine in British Columbia that is becoming more prevalent and has the potential to become a public health issue. Bromazolam is commonly detected in the local drug supply with fentanyl. In 2022, Drug Analysis Service analysis of seized drugs showed an increase only in samples containing bromazolam, fentanyl, and caffeine, which may explain the higher *in vivo* bromazolam concentrations and underscore the need to add it to screening panels. The B.C. Provincial Toxicology Centre reports preliminary results back to the coroner's office within 24 hours. However, by utilizing retrospective analysis, unknown compounds found in circulation at a later date still have the possibility of being identified in the future.

References

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