Novel psychoactive substance detection using a novel multi-aspect workflow solution

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ABSTRACT

Purpose: Demonstrate detection of new and novel psychoactive unknown substances using a multi-aspect untargeted method.

Methods: Human urine samples were spiked with fentanyl analogues not present in reference libraries. Reference spectra for 66 fentanyl analogues were used to make a fragment list for frequently observed fragment ions due to common substructure. This list was used, in combination with spectral library similarity search, to detect compounds of interest in the suspect samples. Lastly, molecular maps were built linking related compounds in suspect urine samples. The compound-class/similarity search compounds were used to direct searching the map for any missed NPS.

Results: Multi-aspect untargeted workflows used on suspect samples can detect NPS with confidence.

INTRODUCTION

Novel psychoactive substances (NPS) are synthetic drugs that mimic existing pharmacological effects of the original drug but at a greater magnitude. In recent years, drug abuse and overdose deaths have risen due to an influx of NPS, comprising of previously unobserved designer drugs. Identifying NPS has been a challenge for forensics as new compounds appear in the market which are missed by traditional targeted analysis approaches. In order to keep up with NPS and detect them quickly, a new approach is required using untargeted analysis tools that can detect these unknowns in complex biological samples. Herein we describe a new data processing approach by using a workflow in Thermo Scientific™ Compound Discoverer™ 3.1 software that utilizes molecular networking in combination with spectral similarity searching using mzCloud™ reference library and compound-class based fragment detection that will help in detecting novel fentanyl analogues.

MATERIALS AND METHODS

Sample Preparation

Stock solutions of six individual pure fentanyl standards were prepared by dissolving the solid in methanol. 'Clean' urine samples were centrifuged and the supernatant was diluted in water (1:1). Three 'clean' human urine samples were spiked with one to three different fentanyl analogue stock solutions.

Mass Spectrometer Acquisition Conditions

Gradient elution was performed on a Thermo Scientific™ Accucore™ Phenyl-Hexyl Column (100 x 2.1 mm, 2.6 µm) at a column temperature of 40 ºC. An LC gradient (Table 1) was done at a flow rate of 0.500 ml/min throughout the acquisition. Ionization was performed by electrospray ionization in positive mode. An initial full scan was set at a resolution of 30,000. Data-dependent MS^2 was triggered using higher energy collisional dissociation (HCD) with a stepped collision of 20, 30, 50 normalized collision energy (NCE) at a resolution of 15,000. A Targeted Mass Exclusion was applied to remove peaks from common urine metabolites.

Mass spectrometer:	Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mass Spectrometer
LC:	Thermo Scientific™ Vanquish™ UHPLC system

Table 1. LC Gradient for Sample Analysis

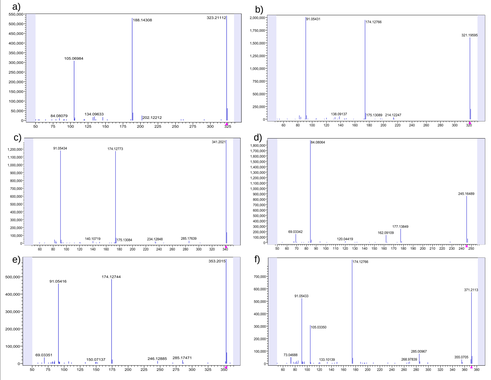
Time (min)	% A (Water + 0.1% Formic Acid)	% B (MeOH + 0.1% Formic Acid)
0	99	1
1	99	1
10	1	99
11.5	1	99
11.51	99	1
15.50	99	1

RESULTS

Multi-Aspect Workflow Overview

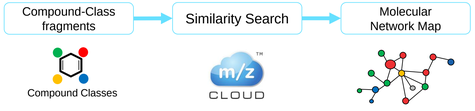
In this study, six different fentanyl analogues were used and spiked in human urine samples. HRAM MS/MS data were acquired on 'clean' urine samples to identify human urine metabolites. These ID's were used as an exclusion list for analysis of the suspect samples. All fentanyl analogues acquired MS^2 spectra (figure 1) with similar fragments among one another.

Figure 1. MS^2 fragmentation data on 6 fentanyl analogues a) Acetyl Fentanyl, b) Benzyl Acryl fentanyl, c) N-Benzyl-meta-fluoro-norfentanyl, d) Cyclopropyl norfentanyl, e) N-benzyl-para-fluoro-cyclopropyl norfentanyl, f) N-benzyl-phenyl norfentanyl



To overcome limitations in current targeted workflows that are used to identify known designer drugs, an untargeted workflow used in data processing software is needed to identify NPS that are not in any reference libraries. Using this approach, data analysis consisted of three independent processes (figure 2) that utilize class-base common fragment ions, similarity search using spectral data found in reference libraries, and a molecular network that links one compound to another that differ by some transformation such as methylation, hydration, dealkylation, etc.

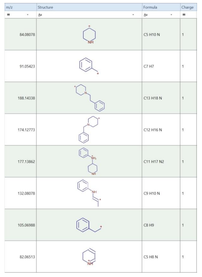
Figure 2. Multi-Aspect workflow



Compound-Class Fragments

The first component of this multi-aspect workflow approach relied on the construction of a set of common fragments for known fentanyl analogues. To do this, reference spectra for 66 fentanyl analogues were used to make a fragment list for frequently observed fragment ions. Due to common substructures, similar fragments (Figure 3) are observed and a frequency distribution was used to select a set of 8 highly overlapping fragment ions. These fragment ions were added as a user-defined compound class library to determine the probability of matching fragments for unknown compounds.

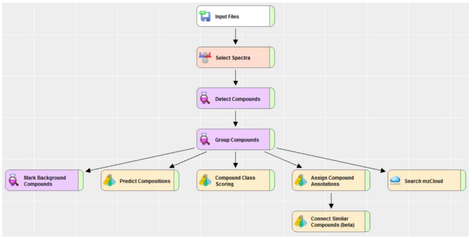
Figure 3. List & Libraries - Common Fentanyl Fragments Display



Compound Discoverer Workflow Overview

An untargeted NPS workflow was used for processing of the raw data and compound ID. Figure 4 shows the nodes used for this process which includes nodes for Compound Class Scoring, Search mzCloud, and Connect Similar Compounds. Compound Class Scoring uses selected fragment libraries set by the user and then scores the similar fragments per compound. Similarity search utilizes the mzCloud spectral reference library to provide useful structural information. Lastly, the Connect Similar Components node is used to create a molecular networking map which shows how detected components are related to each other based on selected transformations.

Figure 4. Compound Discoverer 3.1 software – Workflow Display



Compound-Class Search

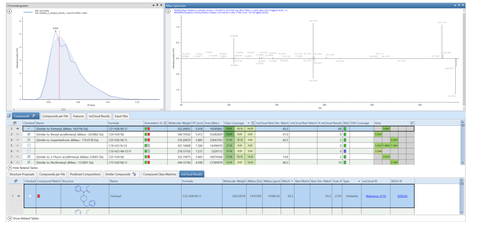
Class-based fragment searches use information from fragments of known compounds to find unknown compounds with related fragments. This untargeted peak detection on suspect samples was processed to flag compounds which contained at least one of the fragment ions set in the compound class scoring node and the resulting peaks were sorted to provide those with the highest occurrence of fentanyl class fragments. On average 5 to 10 compounds were found in each suspect urine sample having 25% or more common fragment ions from a total untargeted peak list of over 4,500 urine components. This resulted in significant reduction of complexity for finding potential NPS, but was not sufficient alone as false positives were still present. Fentanyl analogue metabolites were mixed in the group of false positives because less common fragments were found for these compounds that did not have major core fentanyl fragments, such as in norfentanyl metabolites.

Figure 5. Compound Discoverer 3.1 software - Compound-Class Search Display

Similarity Search Results

In the Search mzCloud node, the settings were set to search through compounds that are under Drugs of Abuse/Illegal Drugs category along with a similarity forward search. The query data includes spectra acquired by stepped collision energies and when using the similarity search mzCloud node the software is capable of utilizing stepped collision energy scans from multiple energy levels of fragmentations to model matches. In figure 6, the query spectra (top) is shown for the selected compound ($C_{23}H_{30}N_2O$). The reference library spectra (bottom) shows an *in silico* average representation spectra based on the collision energies of the query data. The best similarity match for that compound was fentanyl with a mass difference of approximately 14, which corresponds to the formula $C_{22}H_{28}N_2O$. The selected compound is the standard acetyl fentanyl, which differs from fentanyl by a methylene group, a mass difference of about 14. This was also useful is finding similar norfentanyl metabolites in suspect samples that compound-class search alone could not identify as a potential fentanyl analogues. By combining the compound-class fragment results with parallel spectral library similarity search, this provided a finer set of results in which only 1 to 5 compounds of interest were found in suspect urine samples.

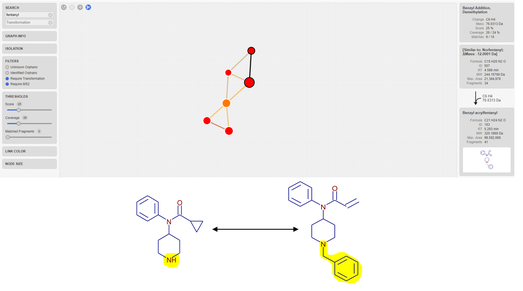
Figure 6. Compound Discoverer 3.1 software - Similarity Search Display



Connecting Similar Compounds

A molecular map for the untargeted urine components was generated which linked components based on spectral similarity, overlap, or any of a list of potential formula changes. These preset transformations can be selected through this node along with manually added possible transformation, in this case transformations specific to fentanyl analogues. The molecular network map shown in Figure 7 was created with peaks identified through the combined compound-class and similarity as starting points to find potential NPS missed by the other approaches alone. This approach was able to identify and connect cyclopropyl norfentanyl, a norfentanyl metabolite, with another compound that was matched through the search mzCloud node that differed by a benzyl addition transformation.

Figure 7. Molecular Networking Map Display – Connection between two compounds with a benzyl addition transformation difference



Overall, the use of each of these processing nodes resulted in detecting each fentanyl, either individually or in combination with one another. The class-base search was able to detect potential fentanyls with highly matching fragment ions. This approach alone also detected 1-2 false positives, which were resolved by also utilizing similarity search to see if those compounds were similar to other fentanyl analogues in the reference library. In the case that a compound showed a high score from having similar ions, but similarity search did not result in detecting it as a potential fentanyl analogue, a molecular map can be used to deduce if that compound is in some way related to other fentanyls in the suspect samples, but differ by some transformation. This untargeted workflow is a unique approach that detected fentanyl analogues not found in any reference library that could not have been detected by traditional targeted approaches.

CONCLUSIONS

- In each of the spiked suspect urine samples, all fentanyl analogues were detected using this workflow. False positive compounds were also provided as potential compounds of interest in 2 of 3 total samples with either one or two false positive components present above the criteria.
- The structure information for fragment ions provided from the compound-class list, and the structure similarity data from the spectral library was combined to build putative structures for the NPS
- Data processing software with multi-aspect identification methods is useful in untargeted NPS detection.

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TRADEMARKS/LICENSING

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