Simultaneous Quantitation and Discovery (SQUAD): A Combination of Targeted and Untargeted MS Workflows for Drug Toxicology

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Abstract

Purpose: Demonstrate an intelligent data acquisition workflow for LC-MS clinical toxicology with deep metabolome coverage, accurate metabolite quantitation, and confident compound annotation in human urine.

Methods: A 15.5-min analytical method was developed on the Thermo Scientific™ Vanquish™ Horizon coupled with Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer, targeting 64 specific novel psychoactive substances (NPS) with Thermo Scientific™ TraceFinder™ Software 5.1. The data was then analyzed using an untargeted approach using Thermo Scientific™ Compound Discoverer™ 3.3 software.

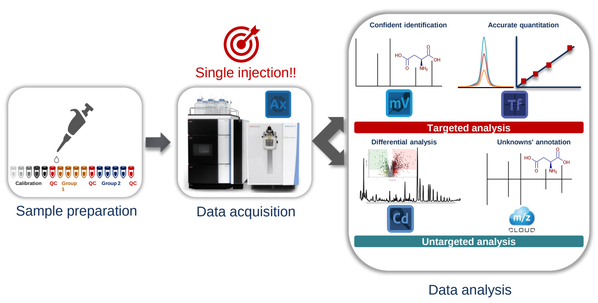
Results: In the pooled patient samples, a number of fentanyls and other NPS were quantitated and a total of six fentanyl analogues were identified and compared between the mixes.

Introduction

As drug abuse continues to be on the rise, it is important to be able to identify drugs and their metabolites in toxicological samples. Without internal standards and libraries, untargeted metabolomics lacks accurate quantitation and identification of metabolites needed to study biological systems. These steps can complicate the study design and data processing; thus, many researchers prefer to target a few analytes and risk missing significant compounds.

Therefore, a single-injection simultaneous quantitation and discovery (SQUAD) metabolomics workflow that provides confident identification and/or accurate quantitation of analytes such as drugs, by analyzing their authentic standards, without compromising the untargeted analysis is preferred. The workflow also enables the discovery of analytes with potential biological significance like drugs' metabolism products (Figure 1). The goal of this study was to use SQUAD analysis to uncover the presence and relative quantitation of drug metabolites present in unknown urine samples.

Figure 1. Illustration of the SQUAD toxicology workflow.



Materials and methods

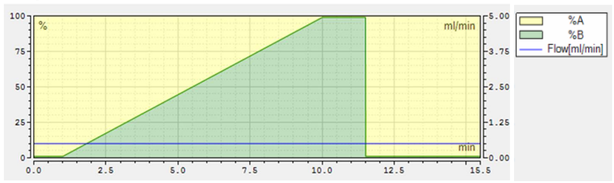
Sample Preparation

14 unknown urine patient samples and a calibration curve set containing 64 novel psychoactive compounds, obtained from Quest Diagnostics, were hydrolyzed and then diluted in mobile phase. These samples were pooled into three different mixes.

Liquid Chromatography

Analytes were separated on a Thermo Scientific™ Vanquish™ Horizon ultra-high performance liquid chromatography (UHPLC) system equipped with a Thermo Scientific™ Accucore™ phenyl hexyl, 100 x 2.1 mm, 2.6 µm column. Mobile phases were 2 mM ammonium formate with 0.1% formic acid in (A) water and (B) methanol:acetonitrile (1:1), and run with the gradient in Figure 2.

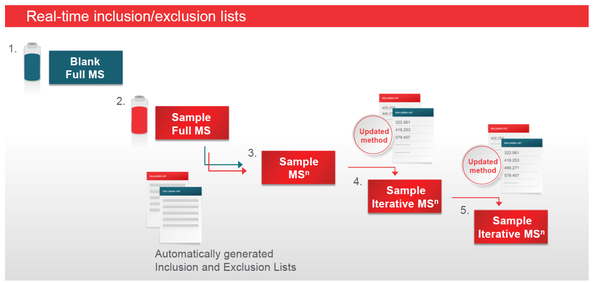
Figure 2. UHPLC Chromatographic gradient used for data acquisition.



Mass Spectrometry

Untargeted screening and quantitation were performed on a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer. Resolutions of 60,000 (FWHM at m/z 200) for full scan and 15,000 for MS2 were employed. An isolation window of m/z 1.5 and stepped collision energies (18.75, 37.5, 56.25) were applied to generate rich HRAM MS2 spectra. AcquireX ™ Deep Scan was used to create iterative data acquisition off of an automatically generated inclusion list.

Figure 3. Thermo Scientific™ AcquireX Deep Scan mode for intelligent data acquisition to maximize the number of relevant compounds interrogated by MS/MS, resulting in higher coverage and confidence annotation.



Data Analysis

Post-acquisition data analysis was carried out using Thermo Scientific™ TraceFinder™ software (v. 5.1) for quantitation and Compound Discoverer™ 3.3 for untargeted analysis. Within Compound Discoverer™ the following tools were used to help identify compounds: delta ppm, mzCloud, Chem Spider, a Drugs of Abuse mzVault library, Class Coverage (compound class fragment library), and peak ratings. Class Coverage allows for the ability to add the common fragments of a drug class in order to make identifications of potential compounds that may reside within a specific drug class. A Class Coverage was created for common fentanyl fragments (Figure 4).

Figure 4. Class Coverage for common fentanyl fragments.

	m/z	Structure	Formula	Charge
1	84.08078		C5 H10 N	1
2	91.05423		C7 H7	1
3	188.14338		C13 H18 N	1
4	174.12773		C12 H16 N	1
5	177.13863		C11 H17 N2	1
6	132.08078		C9 H10 N	1
7	105.06988		C8 H9	1
8	82.06513		C5 H8 N	1

Results

Quantitation

Within the 3 mixes, several compounds present in the calibration curve were detected. These compounds were quantitated and their respective concentrations in each mix are presented in Table 1. N,N-dimethylpentylone, a novel stimulant, had significantly high concentrations in 2 of the 3 mixes, and fentanyl and norfentanyl were present in all 3 mixes.

Table 1. Heat map of the calculated concentrations in ng/mL for several of the compounds detected from each of the 3 mixes.

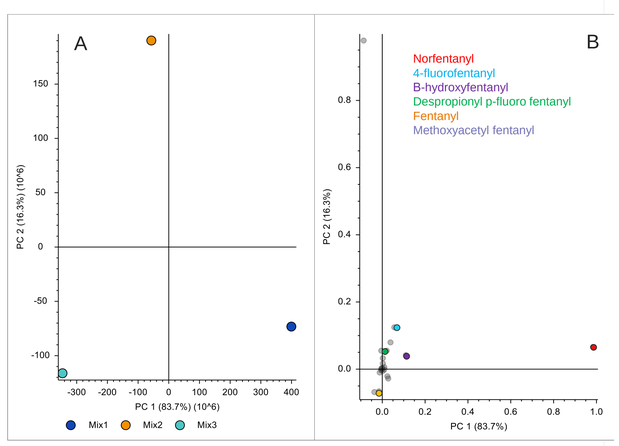
	Mix 1	Mix 2	Mix 3
4-Fluorofentanyl	106.2	116.7	0.0
a-Pyrrolidinohexanophenone (a-PHP)	41.0	0.0	0.0
Fentanyl	179.9	159.1	212.6
N,N-Dimethylpentylone	2376.8	0.0	4835.3
Norfentanyl	2828.6	1409.0	699.6
Pentylone	1774.6	0.0	8555.7

Untargeted Analysis

By utilizing an untargeted approach, compounds beyond a set inclusion list and calibration curve are able to be identified. Here we show how to analyze differences between the mixes to show important features or drug metabolites from different groups and how to best use the filter tools in Compound Discoverer to narrow a search to look for unknown drug metabolites.

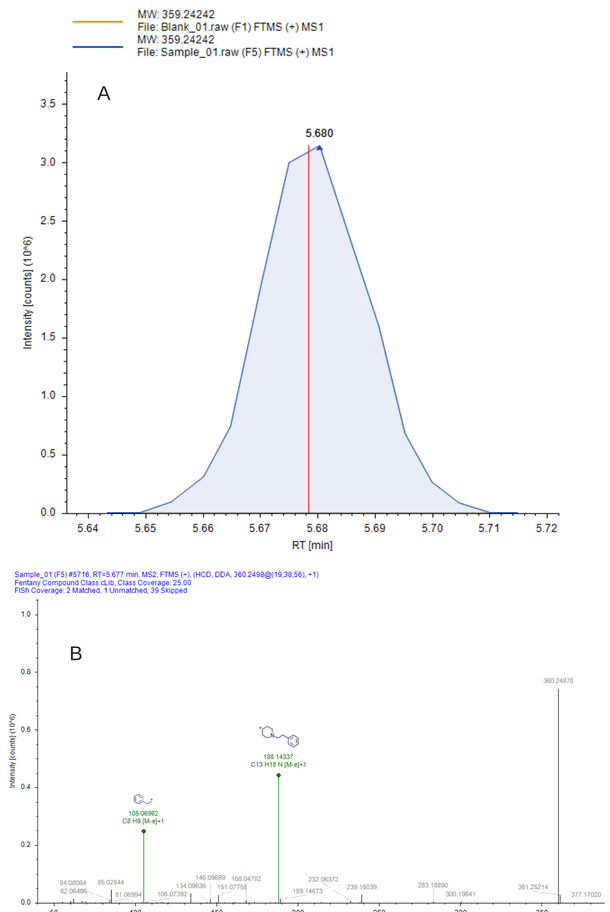
Through a PCA analysis, differences between each group's metabolite concentrations can be observed (Figure 5). For example, the PCA plot demonstrates how norfentanyl had a greater presence in Mix 1 than Mix 2 or 3 and despropionyl p-fluoro fentanyl, an impurity in the synthesis of para-fluorofentanyl, had relatively similar concentrations in Mixes 1 and 2 but was not present in Mix 3.

Figure 5. Data from the 3 mixes presented in the A) scores plot and B) loadings plot of PCA analysis showing differences between six fentanyl analogues in the three groups.



Applying compound filters in Compound Discoverer is an excellent way to narrow down results of batched data or specific samples to find potential unknowns. By using filters of +/- 5 ppm mass accuracy and class coverage scoring of 12.5 (1 fentanyl fragment or greater identified), we were able to determine potential fentanyl analogues in the sample. During this study, an unnamed compound with no confirming ID's from mzCloud, Chem Spider, or the mzVault library was made (Figure 6). This unknown compound had a retention time of 5.68 minutes, which is similar to fentanyl compounds/metabolites. A fentanyl compound class score of 25 indicated the software detected 2 fragments that match common fentanyl fragments. More research would need to be done to determine the exact identity of this compound.

Figure 6. Unnamed compound represented by it's A) extracted ion chromatogram and B) fragmentation spectrum.



Conclusions

The SQUAD toxicology workflow enables the ability to perform targeted and untargeted analysis of a single-sample injection of biological samples. This workflow will allow the identification of new drugs and their metabolites while targeting a list of frequently tested dug metabolites.

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