Forensic Identification of Unknown Compounds in Human Urine Using Complementary Software with High-Resolution Data and Theoretical Fragmentation Spectra

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INTRODUCTION

Identification of novel psychoactive substances (NPS) present challenges to forensic toxicologists, particularly when the compounds are not commercially available and corresponding fragmentation spectra do not exist in either commercial or lab-based libraries.

This poster presents a new workflow that facilitates identification and confirmation of such compounds when information is limited to only molecular formula and structure. The workflow leverages the power of complementary data analysis software packages and high-resolution mass data to provide confident compound identification.

MATERIALS AND METHODS

Sample Processing

- Urine samples were spiked with test compounds at 100, 10 and 1 ng/mL and then diluted 20-fold with water.

Liquid Chromatography

- Thermo Scientific™ Dionex™ UltiMate 3000™ HPG-3400RS pump with OAS-3300TXRS autosampler.
- Mobile Phase A: 5 mM ammonium formate with 0.1% formic acid in water
- Mobile Phase B: 5 mM ammonium formate with 0.1% formic acid in methanol
- Column: Thermo Scientific™ Accucore™ Phenyl-Hexyl, 2.6 µm, 50 x 2.1 mm
- Gradient: 3-95% B in 8 minutes, 11 minutes total run time

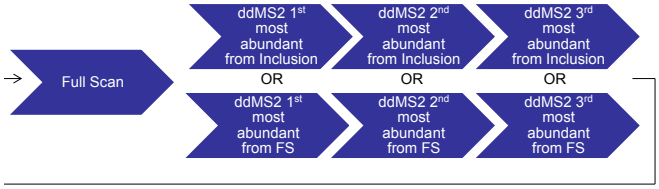
Mass Spectrometry

- Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer
- HESI ionization source

Data Acquisition

- Full scan (FS) MS spectra at a resolution of 70,000 (FWHM at *m/z* 200)
- Data-dependent MS-MS fragmentation (ddMS2) spectra at a resolution of 17,500 (FWHM at *m/z* 200)
  - ddMS2 triggered on compound *m/z* from inclusion list
  - If no *m/z* from inclusion list is detected in FS, ddMS2 triggered for most abundant *m/z* detected in FS (**Figure 1**)
  - An exhaustive exclusion list was used to prevent ddMS2 collection for irrelevant matrix components *m/z*'s

Figure 1. Schematic of Data Acquisition Method



Full scan spectra were acquired followed by three ddMS2 spectra triggered on either masses from an inclusion list or, if no mass from the list was found, the most abundant masses detected in the full scan. An exclusion list of endogenous matrix components was used to prevent acquisition of irrelevant fragmentation spectra for matrix background.

Data Processing

Data were acquired and processed with Thermo Scientific™ TraceFinder ™ (version 4.0) software. Schematic of data processing workflow is presented in **Figure 2**.

- In the first phase of the data processing workflow, the software detected all chromatographic peaks above the threshold specified in the method.
- Next, the detected peaks were identified based on accurate mass and isotopic pattern using a user-created database. The only information on this database was the molecular formula and accurate *m/z*+. Since it was assumed that the compounds were novel unknowns and there were no standards in-house, no spectral library was used, and no retention times were in the database.
- Next for every detected peak, TraceFinder-proposed up to three (set in method) molecular formulae for each peak based on the extracted accurate mass and isotopic pattern of the peak. The highest ranked molecular formula was sent to the ChemSpider™ search tool which returned three (set in method) possible molecular structures.
- In the next phase of the workflow, Mass Frontier™ software (version 7.0, HighChem) was used to generate theoretical fragmentation spectra for the ChemSpider hits and compare them to experimental fragmentation for the related chromatographic peak to select and confirm the best hit.

Figure 2. Data Processing Workflow

Data Processing Step	Software Package
Detect all chromatographic peaks above threshold	TraceFinder version 4.0
⇩	⇩
Search Full Scan data against user created database containing molecular formula and *m/z*.	TraceFinder version 4.0
⇩	⇩
Generate possible molecular formula from accurate mass and isotopic pattern.	TraceFinder version 4.0
⇩	⇩
Search that molecular formula in ChemSpider databases for possible structures.	TraceFinder version 4.0
⇩	⇩
Confirm molecular structure returned by ChemSpider with theoretical fragmentation spectra.	Mass Frontier version 7.0

Method Evaluation

In order to evaluate this workflow, fentanyls (**Figure 3**) were spiked into human urine at 100, 10 and 1 ng/mL. Samples were processed and analyzed as previously described.

Method performance was evaluated based on its ability to identify spiked analytes.
Specific elements of the workflow assessed were:

- Ability to correctly detect compounds listed in user-created database.
- Accuracy of proposed molecular formulas.
- Ranking of ChemSpider structure hits.
- Confirmation of hits by theoretical fragmentation in Mass Frontier.

RESULTS

All compounds were detected at the lowest evaluated concentration of 1 ng/mL except sufentanil which was detected at 10 ng/mL. All compounds had perfect isotopic pattern scores at 100 and 10 ng/mL. At 1 ng/mL, nine of the compounds had passing isotopic pattern scores and seven had lower scores. This is not unexpected because of the lack of response for the lower abundant isotopes.

Since molecular formula is based on accurate mass and isotopic pattern, the results for the molecular formulae proposed by TraceFinder software followed the same pattern as those for isotopic pattern matching. Experimental data for isotopic masses is required for accurate prediction of molecular formula. If the isotopic pattern score was high, the molecular formula was more likely to be accurate. The correct formula was the top-ranked result proposed for all 100 ng/mL samples and all but one (W-15) of the 10 ng/mL samples. At the 1 ng/mL level, half of the compounds had a correct molecular formula as the top ranked hit; 25% had the correct formula as the second ranked hit; the remaining 25% did not generate the correct molecular formula in the top three, which was the limit set in the method. This is again explained by low abundance of isotopic mass signal in 20-fold diluted urine samples since isotopic pattern is used to predict molecular formula.

Figure 4 shows results for representative analyte W-15 at 100 ng/mL. The correct molecular formula was the top hit calculated from the exact mass and isotopic pattern. The top three ChemSpider search results are listed, and for those results, theoretical versus experimental fragmentation was compared to select the best hit.

Results for all compounds are summarized in **Table 1**.

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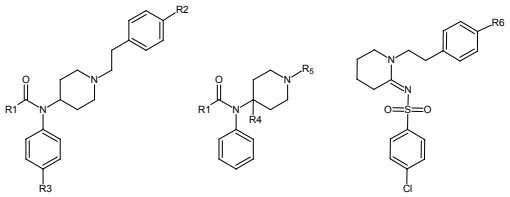
The ability of ChemSpider to return the correct structure as the first result varied depending on the databases selected along with the number of references within those databases. Because these synthetic fentanyls are relatively new, they have fewer references in the ChemSpider databases and two of the compounds (furanylfentanyl and 4-methoxybutyrylfentanyl) had no references at all. The lack of references resulted in lower ranking or no result from the ChemSpider search as demonstrated with data presented at **Figure 4**.

Both lack of results and multiple results from ChemSpider is why confirmation using fragmentation spectra proved valuable. Since it was assumed that no reference standard were available for these NPSs, theoretical fragmentation spectra were the only spectra available for comparison.

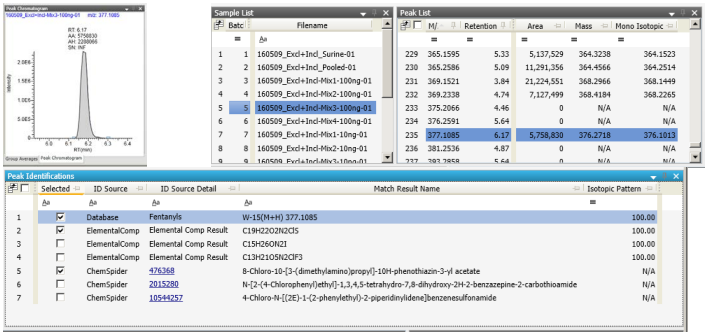
Comparing the theoretical fragmentation spectra with the experimental spectra allowed confirmation of user database-based identity when no ChemSpider database search possibilities were returned (**Figure 5**) and also selection of the most probable structure from those returned by ChemSpider (**Figure 6**).

Figure 3. Fentanyls used for workflow evaluation



Compound	Formula	m/z	R1	R2	R3	R4	R5	R6
4-Methoxy-butyrylfentanyl	$C_{24}H_{32}N_2O_2$	381.2537	$-CH_2CH_2CH_3$	-H	$-OCH_3$	-	-	-
Acetylfentanyl-4-methylphenyl analog	$C_{22}H_{28}N_2O$	337.2274	$-CH_3$	$-CH_3$	-H	-	-	-
Butyrylfentanyl	$C_{23}H_{30}N_2O$	351.2431	$-CH_2CH_2CH_3$	-H	-H	-	-	-
Fentanyl	$C_{22}H_{28}N_2O$	337.2274	$-CH_2CH_3$	-H	-H	-	-	-
Furanylfentanyl	$C_{24}H_{26}N_2O_2$	375.2067		-H	-H	-	-	-
Isobutyrylfentanyl	$C_{23}H_{30}N_2O$	351.2431	$-CH(CH_3)_2$	-H	-H	-	-	-
4-Fluorobutyryl-fentanyl	$C_{23}H_{29}FN_2O$	369.2337	$-CH_2CH_2CH_3$	-H	-F	-	-	-
Valerylfentanyl	$C_{24}H_{32}N_2O$	365.2587	$-(CH_2)_3CH_3$	-H	-H	-	-	-
Acetylnorfentanyl	$C_{13}H_{18}N_2O$	219.1492	$-CH_3$	-	-	-H	-H	-
Norfentanyl	$C_{14}H_{20}N_2O$	233.1648	$-CH_2CH_3$	-	-	-H	-H	-
Norsufentanil	$C_{16}H_{24}N_2O_2$	277.1911	$-CH_2CH_3$	-	-	$-CH_2OCH_3$	-H	-
Alfentanil	$C_{21}H_{32}N_6O_3$	417.2609	$-CH_2CH_3$	-	-	$-CH_2OCH_3$		-
Sufentanil	$C_{22}H_{30}N_2O_2S$	387.2101	$-CH_2CH_3$	-	-	$-CH_2OCH_3$		-
β-Hydroxythio-fentanyl	$C_{20}H_{26}N_2O_2S$	359.1788	$-CH_2CH_3$	-	-	-H		-
W-15	$C_{19}H_{21}ClN_2O_2S$	377.1085	-	-	-	-	-	-H
W-18	$C_{19}H_{20}ClN_3O_4S$	422.0936	-	-	-	-	-	$-NO_2$

Figure 4. Results from TraceFinder software for W-15 at 100 ng/mL.



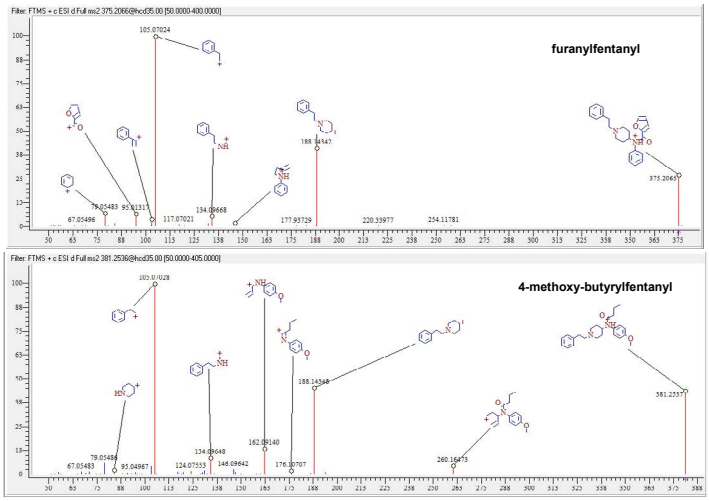
Top Left: Peak for mass 377.1085 extracted from the full-scan data at a mass tolerance of 5 ppm.
Top Right: Integration results for the identified peak.
Lower: Peak identification results showing user database hit, elemental composition (molecular formula) results and the top three ChemSpider hits. The correct molecular formula was generated, and the third ChemSpider hit is the correct structure.

Table 1. Results from TraceFinder Software

Compound	LOD (ng/mL)	Isotopic Pattern Score %			Formula Rank		
		100 ng/mL	10 ng/mL	1 ng/mL	100 ng/mL	10 ng/mL	1 ng/mL
4-Methoxybutyrylfentanyl	1	100	100	0	1	1	0
Acetylfentanyl-4-methylphenyl analog	1	100	100	100	1	1	1
Butyrylfentanyl	1	100	100	14	1	1	0
Fentanyl	1	100	100	100	1	1	1
Furanylfentanyl	1	100	100	100	1	1	1
Isobutyrylfentanyl	1	100	100	100	1	1	2
4-Fluorobutyrylfentanyl	1	100	100	14	1	1	0
Valerylfentanyl	1	100	100	100	1	1	2
Acetylnorfentanyl	1	100	100	14	1	1	1
Norfentanyl	1	100	100	100	1	1	1
Norsufentanil	1	100	100	100	1	1	1
Alfentanil	1	100	100	100	1	1	1
Sufentanil	10	100	100	0	1	1	0
β-Hydroxythiofentanyl	1	100	100	100	1	1	1
W-15	1	100	100	64	1	3	0
W-18	1	100	100	0	1	1	0

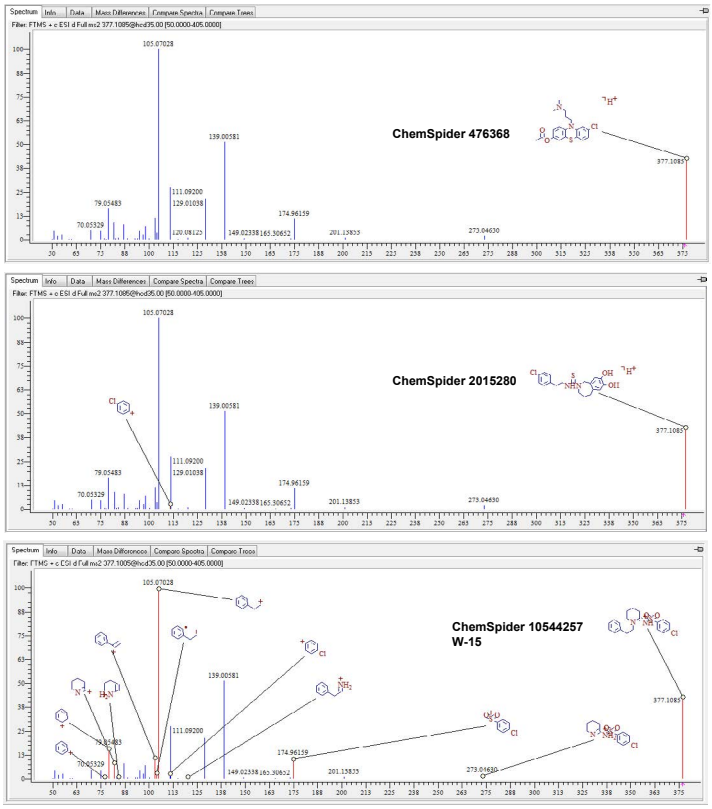
Results from analysis of synthetic fentanyls in 20-fold diluted urine showing Limit of Detection (LOD), Isotopic Pattern Matching Score and the rank of the correct molecular formula returned by the software. Isotopic pattern matching scores were poorer at the lowest concentration which is to be expected due to the corresponding lower response of the lesser abundant isotopes.

Figure 5. Mass Frontier software: annotation of fragments in experimental spectra using data obtained by theoretical fragmentation for furanylfentanyl and 4-methoxy-butyrylfentanyl.



The structures for furanylfentanyl and 4-methoxybutyrylfentanyl (Figure 3) were subjected to theoretical fragmentation in Mass Frontier software and matched to the corresponding experimental fragmentation spectra. Such matching can provide confidence in identification of a novel compound when no reference standards are available and no spectra exist in spectral libraries.

Figure 6. Mass Frontier software: annotation of fragments in experimental spectra using data obtained by theoretical fragmentation for 3 possible structures for specific molecular formula.



Each of the three molecular structures returned by ChemSpider search tool for W-15 hit (**Figure 4**) were subjected to theoretical fragmentation in Mass Frontier software and matched to the corresponding experimental fragmentation spectra. The first structure matched no fragments, only the precursor mass. The second structure had one fragment match in addition to the precursor. The third structure, which is the correct structure for W-15, returned 12 matches between the theoretical and experimental fragmentation.

CONCLUSIONS

- We demonstrated LC-MS workflow allowing identification of NPSs in biological matrix for which available information is limited to chemical structure and formula.
- The workflow was demonstrated using synthetic fentanyls spiked into pooled donor urine.
- Accurate calculation of proposed molecular formula of results depends on intensity of isotopic abundance in full scan spectra.
- Careful selection of appropriate ChemSpider databases will enhance the ranking of possible structures.
- Theoretical fragmentation spectral matching provides confidence in identification of compounds when no standards or spectra exits in-house.

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