Pioneering Tool to Characterize Emerging Fentanyl Analogues

Implementing a Non-Targeted Screening Workflow with the SCIEX X500R QTOF System

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The continuous abuse of fentanyl and its derived analogue substances continues to be a serious public health and safety problem. Deaths related to overdose following ingestion of fentanyl and its analogues are on the rise, as more of these compounds emerge into the street drug supply [1-2].

Fentanyl analogues are compounds clandestinely synthesized to produce similar psychotropic effects to that of fentanyl. These analogues are often produced with slightly different molecular structures, which makes traditional screening approaches (e.g., Immunoassay or GC/MS) challenging for the investigator. High Resolution Mass Spectrometry (HRMS) provides the forensic examiner a reliable and rapid tool for the analysis of emerging drugs; including opioids, fentanyl, and analogues. Furthermore, the presence of HRMS in the forensic laboratory is essential to support field authority investigations by providing more complete chemical characterization (e.g., formulae finding, molecular interpretation) of a potential fentanyl analogue found in a forensic seized drug preparation or biological sample.

In this technical note, a non-targeted screening workflow for the identification of novel fentanyl analogues in forensic biological samples is described. The workflow was streamlined using the SCIEX X500R QTOF System in combination with the SCIEX OS Software formula finder feature and ChemSpider database searching.

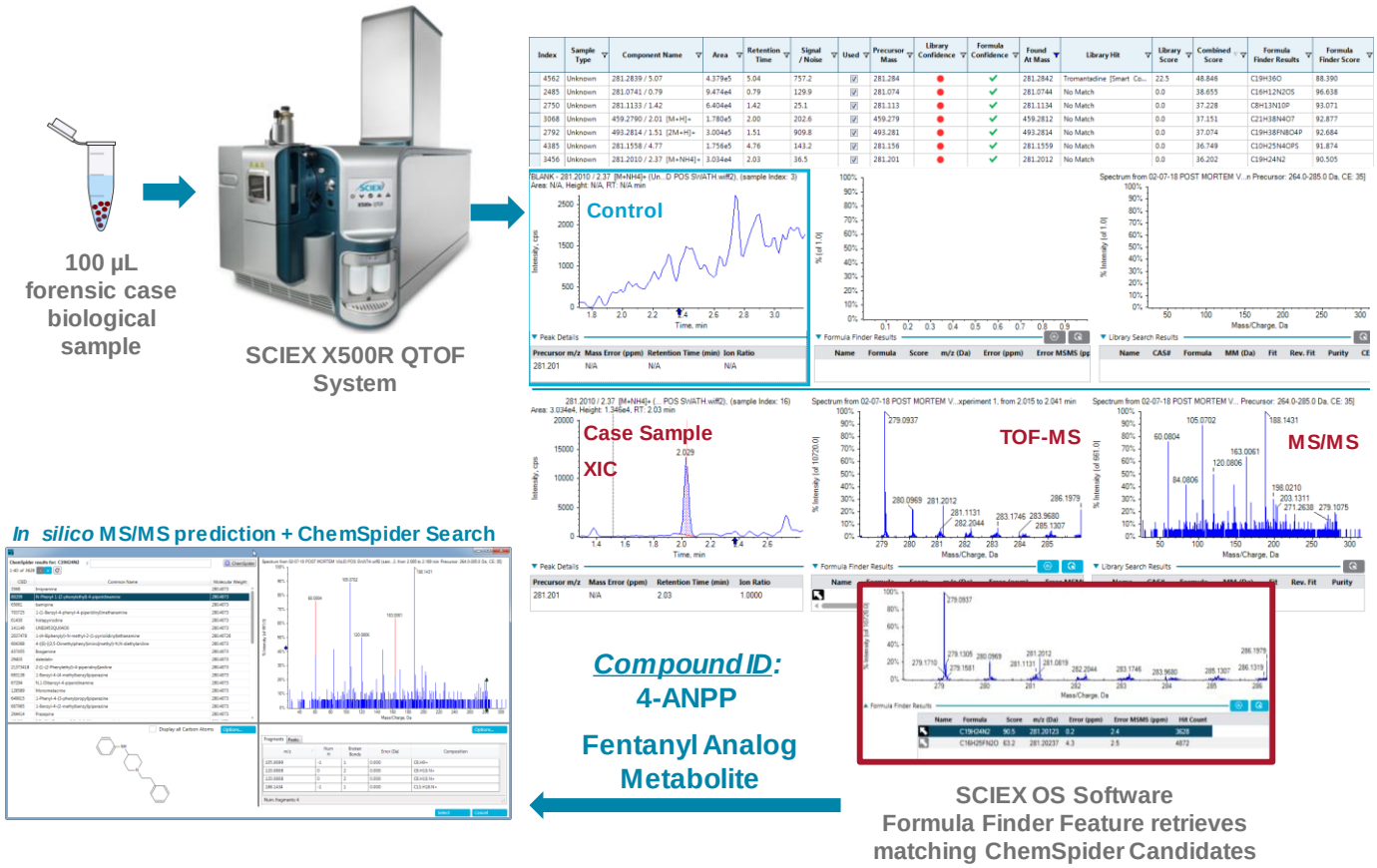

Figure 1. Confidently Identify the Presence of Fentanyl Analogues in an Unknown Postmortem Blood Sample. Data analysis workflow for non-targeted screening in SCIEX OS software. XICs are generated by non-target peak finding algorithms and a sample-control comparison is used to identify relevant and distinct chromatographic features. Empirical formula finding and ChemSpider searching are used to more accurately identify compounds present in a forensic sample. TOF-MS and MS/MS spectra were obtained to enable the identification of 4-ANPP, a precursor and/or metabolite of a fentanyl analogue in a postmortem blood sample.

Features of SCIEX X500R QTOF System for Identifying Novel Fentanyl Analogues

- The X500R QTOF System acquires high resolution MS data to enable the quick identification of drugs of abuse, including fentanyl and its analogues in forensic samples.
- SCIEX OS Software enables the user to quickly identify unknown compounds with greater confidence using integrated library searching capabilities, formula finder, and fragmentation prediction tools to aid in true unknown spectral interpretation and strucutral elucidation.
- The implementation of this revolutionary QTOF technology in combination with SCIEX OS Software empowers the forensic examiner with a streamlined tool to detect every component present in a forensic sample of interest.

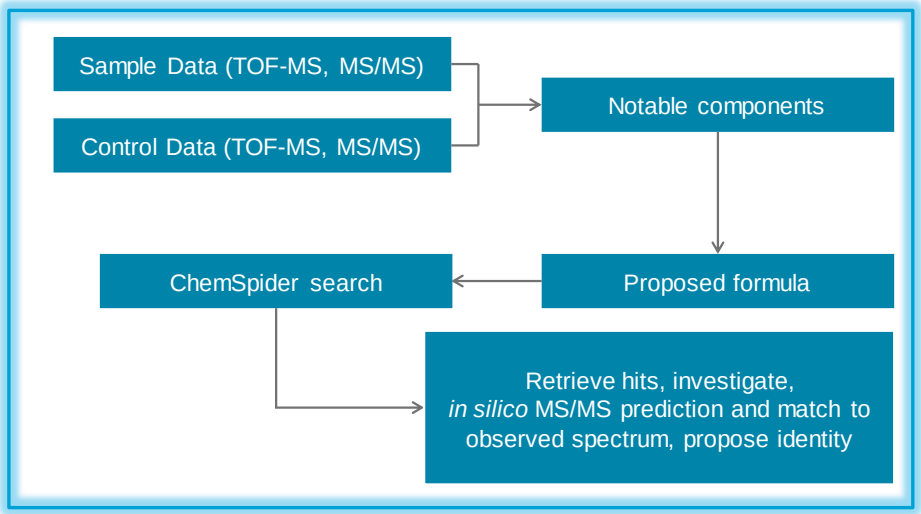

Figure 2. Streamlined Novel Fentanyl Analogue Discovery. The formula finding feature in SCIEX OS Software uses mass accuracy and isotope pattern to calculate possible molecular formulae. Theoretical exact mass fragments are calculated and compared against the MS/MS spectrum to reduce the list of matching formulae. Finally, the software algorithm finds other adduct formations to predict the correct molecular formula of the uncharged analyte, ranking the highest match possible using a combined TOF-MS and MS/MS score.

Experimental Methods

Sample Preparation: Forensic postmortem blood samples were extracted using a protein precipitation, and forensic urine samples were centrifuged and diluted for analysis.

LC-MS/MS Instrumentation: HPLC separation was performed on a Phenomenex Kinetex Phenyl-Hexyl column using the SCIEX ExionLC™ AC system. TOF-MS and MS/MS data were collected using SWATH® Acquisition on the SCIEX X500R QTOF System

Data Analysis: Non-targeted processing was performed using SCIEX OS Software for analyte identification based on two different confidence criteria: Formula Finder Confidence and Library Score. Subsequently, a combined score was computed based on these two confidence categories with custom weighting.

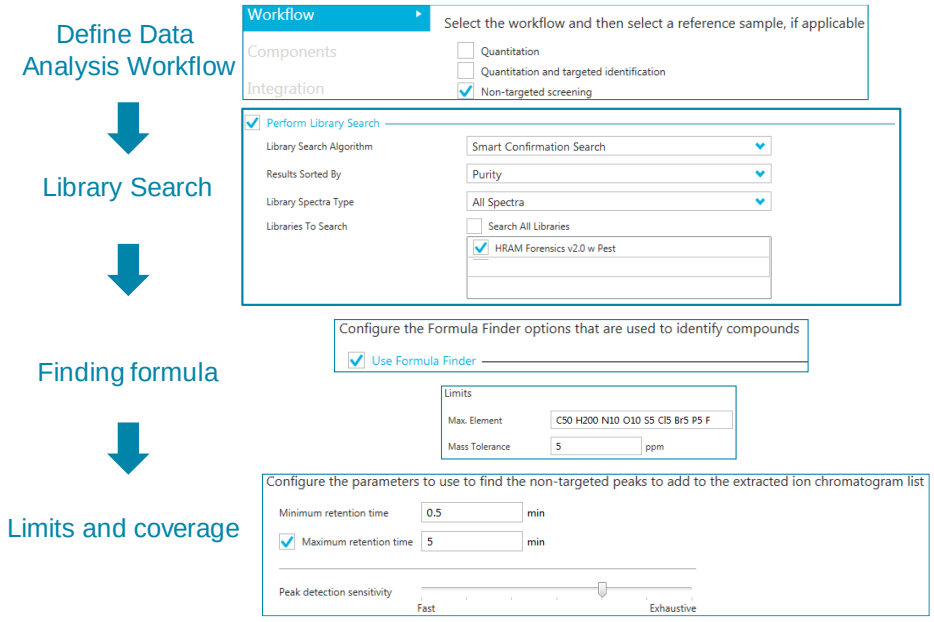

Figure 3. SCIEX OS Software Provides Straightforward Data Analysis Programming. Non-targeted data processing can be easily programmed by defining three parameters: Library Searching algorithms, Formula Finding based on specified elements, and sensitivity of peak detection to retrieve all features present in a forensic sample.

High Quality MS/MS Acquisition Leads to Reliable Fentanyl Analogue Characterization

The understanding of the core molecular structure of fentanyl and its resulting MS/MS fragmentation pattern is a key component in identifying new analogues as they emerge in the field.

Figure 4 shows the structure of Fentanyl ($C_{22}H_{28}N_2O$) and its exact mass MS/MS fragment ions produced. Chemical modifications to fentanyl are often performed by substitutions of or on the phenethyl, piperidine, aniline, and/or propanamide portions of the structure, creating potential positional isomers and structurally variable species, many of which retain the desirable and adverse opioid properties.

These modifications subsequently complicate the characterization process [3], but become extremely useful for the determination of analogue core structure present, due to reproducible and predictable fragmentation patterns.

The SCIEX X500R QTOF system generates comprehensive and high-quality MS/MS spectra, which enables compound fragmentation for the confident identification of novel opioids fentanyl analogues. Figure 5 shows the XIC and TOF-MS spectra of a forensic urine sample suspected of containing a fentanyl analogue.

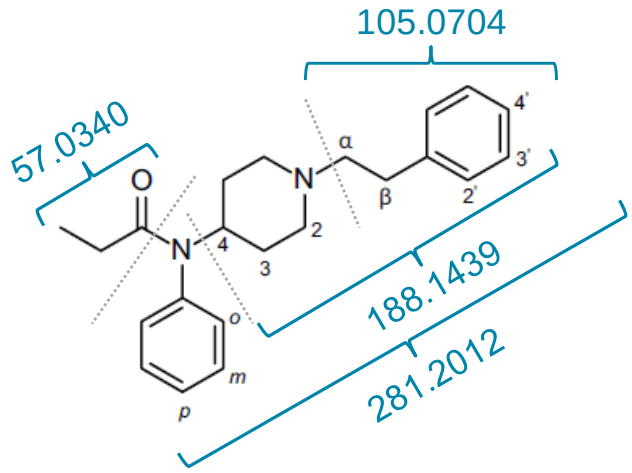

Figure 4. Fentanyl Core Produces Key MS/MS Fragments for Analogue Characterization and Identification. Four fragment ions have been characterized as key components for the identification of emerging fentanyl substances in forensic samples.

An intense peak was found at 2.56 min (371.2128 Da). The use of the formula finding feature, based on the isotope pattern generated by the TOF-MS data, enabled the proposition of candidate formulae. The best fitting formula was $C_{22}H_{27}N_2O_2F$, which is similar in element count to fentanyl, suggesting the presence of a fentanyl analogue

The high-quality MS/MS fragmentation generated from SWATH® Acquisition enabled the further identification of the fentanyl analogue in the forensic urine sample.

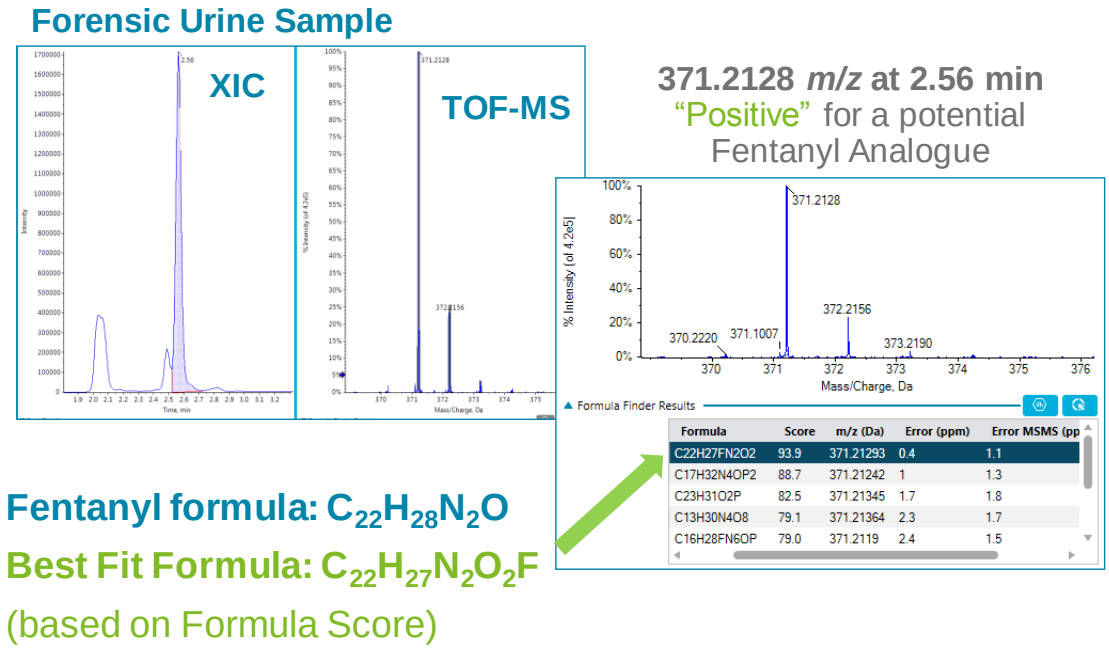

Figure 5. Formula Finder Combines Available Accurate Mass Information to Generate a List of Potential Matching Formulae. A forensic case urine sample analyzed with the SCIEX X500R QTOF System containing a presumptive fentanyl analogue. (Left) Shows the XIC and TOF-MS spectra of a relevant feature, and (Right) formula finder lists the top candidate formulae associated with the peak based on the isotopic pattern obtained.

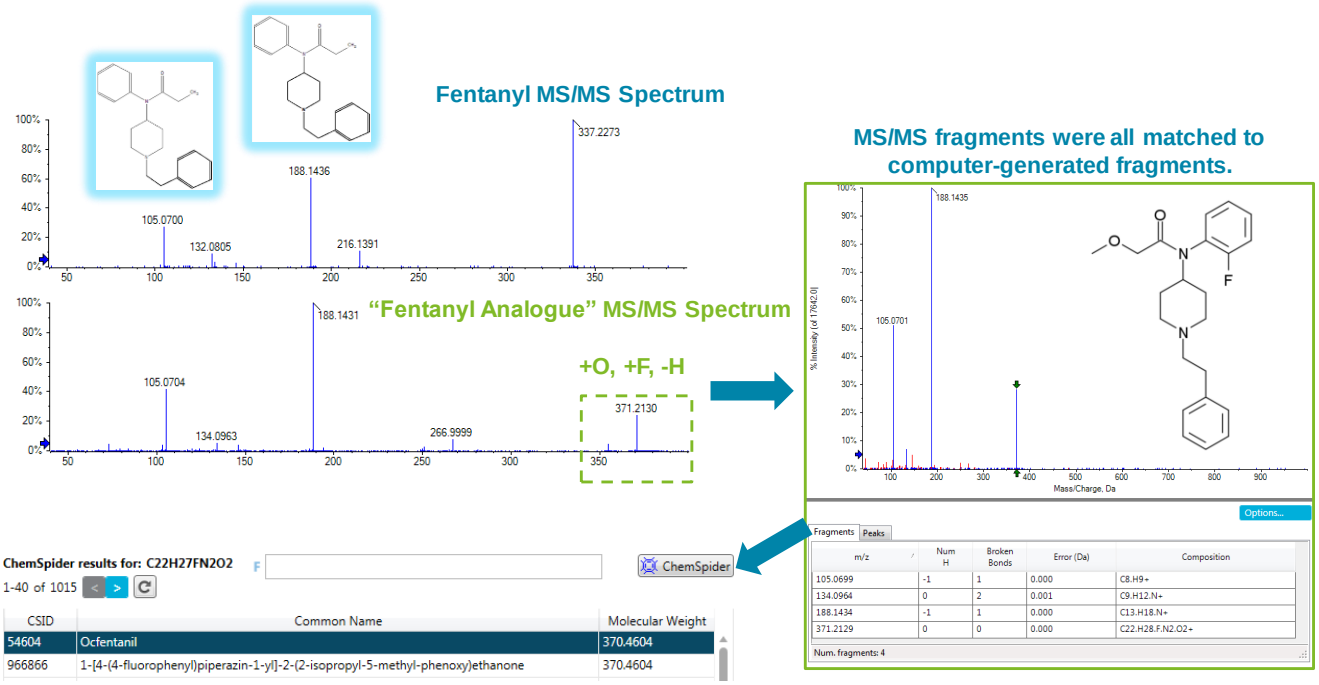

Figure 6. The Combination of SWATH® Acquisition and ChemSpider searching increases compound identification confidence. The differences between MS/MS spectra obtained for Fentanyl (Top) and suspected Fentanyl analogue (Bottom). The *in silico* MS/MS predictions and ChemSpider database searching led to the confident identification of Ocfentanil in this forensic urine sample.

Figure 6 shows the MS/MS spectra comparison between a fentanyl positive sample and the case sample investigated. The 188 m/z and 105 m/z fragments of fentanyl and the *in silico* MS/MS predictions suggest the presence of +F and +O atoms, and removal of one -H atom. The data was further evaluated against the ChemSpider database, which enabled the confident identification of Ocfentanil in this forensic urine sample.

Table 1. Characteristic MS/MS Fragment Ions for Unknown Fentanyl Analogue Identification

Variation of Core Structure**	MS/MS Fragment Ions (Da)		
Fentanyl Core (Propanamide Variants)	281.2012	188.1439	105.0704
α/β-Methyl Substituents	295.2169	202.1596	119.0861
3-Methyl Substituents	295.2169	202.1596	105.0704
α/β-Hydroxy Substituents	297.1962	204.1388	121.0653
	279.1856	186.1283	105.0704
Ortho-, Meta-, Para-Methyl Substituents	295.2169	188.1439	105.0704
Carfentanil (Piperidine Variant)	279.1856	186.1277	105.0704
Thiofentanil (Phenethyl Variant)	287.1577	194.1003	111.0268

These analyte classifications hold true for all fentanyl analogues encounter to date. There are additional core classifications not listed.

Streamlining Fentanyl Analogue Analysis for Postmortem Samples

As new fentanyl related substances continue to emerge in the street drug supply, investigators often rely on their chemistry knowledge to decrypt signs as to what chemical modifications have been made to the fentanyl core structure. The application of HRMS has been a key component to help streamline the characterization of new synthetic opioids [4], including new fentanyl analogues.

Table 1 shows the characteristic MS/MS fragment ions, based on similar core structures, obtained from the individual analysis of different fentanyl analogue reference standards. The characterization of these ions has simplified the drug identification workflow as compounds with common cores provide similar fragmentation patterns.

Hence, the table presented can be used as a guide to determine what core fentanyl-type structure is present, based solely off the identification of these characteristic fragments. For example, if an unknown fentanyl analogue is suspected in a sample and fragment ions 202.1596 Da and 105.0704 Da are present, the analyst can have certainty that this unknown analogue contains the core structure associated with or similar to a 3-methylfentanyl variant.

Additionally, it has been observed that suspected samples containing either α/β-methyl or 3-methyl substituted fentanyl analogues could be distinguished based on MS/MS fragmentation patterns; as α/β-methyl substituents fragment to a 119.0861 Da ion and 3-methyl substituents fragment to a 105.0704 Da ion, due to location of the methyl species and alpha-cleavage of the phenethyl portion during fragmentation.

Figure 7 shows the analysis of the postmortem blood sample suspected of containing a fentanyl analogue, due to the identification of the 4-ANPP metabolite.

A blank control is compared directly against the suspected positive (323.2123 Da, $C_{21}H_{26}N_2O$). The data was further evaluated by generating *in silico* MS/MS prediction and searched against the ChemSpider database, which enabled the identification of acetylfentanyl.

This characterization is supported by the identification of two fentanyl core fragment ions (188.1441 Da and 105.0697 Da). Presence of the 217.0245 Da fragment ion is due to the nature of SWATH® Acquisition (Q1 windowed acquisition), and its relation to the analogue can be ruled out by comparison of ion traces.

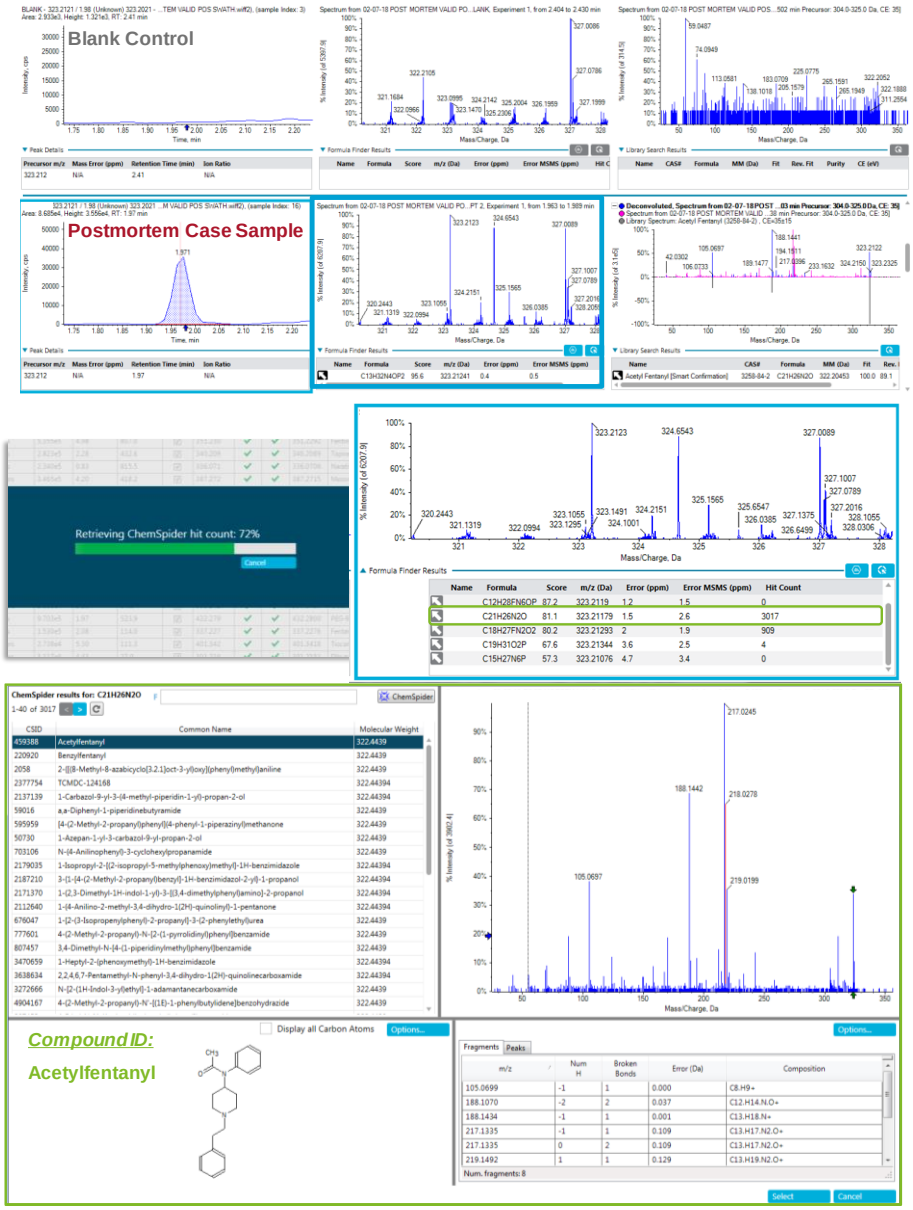

Figure 7. Consolidated Data Processing through SCIEX OS Software. A forensic postmortem blood sample analyzed using the SCIEX X500R QTOF System was automatically processed with a non-targeted screening workflow, which enabled the quick identification of Acetylfentanyl with high confidence in the ChemSpider search results. The *in silico* MS/MS spectral match further affirmed the finding of the fentanyl analog in this case.

Conclusions

- A streamlined non-targeted screening approach for the characterization new Fentanyl analogues was developed and successfully applied to different forensic biological samples using the SCIEX X500R QTOF System.
- The formula finding feature in SCIEX OS Software enabled the characterization of new Fentanyl derived substances (e.g.,Ocfentanil) present in urine and postmortem blood samples, through the generation of in silico MS/MS predictions and search against the ChemSpider Database.
- Characteristic MS/MS fragment ions seen with known Fentanyl analogues have been characterized to provide the forensic investigator a simplified identification workflow as new compounds of this nature continue to emerge.
- Although this workflow enabled the streamlined characterization of emerging fentanyl analogues, the identification of newer unknown fentanyl type compounds will require a thorough MS/MS data examination, if chemical information obtained is not yet housed within a readily available database, such as ChemSpider.

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