Quantitative Analysis of Fentanyl and Analogues in Human Whole Blood

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ABSTRACT

The continuous abuse of fentanyl and its derived analogue substances continues to be a serious public health and safety problem. Death-related 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] As the surge of novel fentanyl analogues continue to flood the street drug market, timely and comprehensive drug screening approaches are critically needed in the forensic laboratory to quickly and accurately identify these novel emerging substances. In this paper, a confirmatory method for fentanyl and its analogues in human blood is described. Using this comprehensive workflow, confident identification of fentanyl, its analogues, and metabolites was achieved in a complex biological matrices such as human whole blood. Quantification of the 29 fentanyl analogues resulted in LODs in the sub ng/mL range while maintaining linearity and precision for all compounds across the calibration range.

INTRODUCTION

Fentanyl analogues and their metabolites are a rising concern as thousands die from opioid overdose across the country. Some of these synthetic drugs have very high potency and thus only require a small amount for an accidental overdose. In addition, these high potency drugs pose a danger to public health and public safety personnel due to the possibility of skin absorption or inhalation of the drug. In order to properly identify these fentanyl analogues in biological matrices, forensic laboratories require sensitive MS systems to accurately quantitate at low concentrations, and highly specific chromatographic methods in order to separate and properly identify isomers.

In this paper, a confirmatory method for fentanyl and its analogues in human blood is described. Due to the inclusion of isomers that are challenging to monitor based on similar fragmentation patterns, the separation of these isomers within the panel was key in method development, in order to accurately identify all fentanyl analogues. The main challenge was separation of these isomers chromatographically while maintaining desirable peak shape and reasonable LC run time. The column used in conjunction with an optimized mobile phase composition produced the separation that was needed to correctly distinguished all isomers. These results demonstrate that the combination of mass spectrometry and highly specific chromatographic methods allows accurate quantification of these new substances while offering confident drug identification at low concentration levels.

MATERIALS AND METHODS

Sample Preparation:
A calibration curve was prepared in methanol and spiked into human whole blood to give desired concentrations ranging from 0.1 ng/ml to 100 ng/ml in order to evaluate the dynamic range. Forensic case samples and controls were extracted for LC/MS screening by using a protein precipitation procedure. In short, 40 µL of methanolic standard solution was spiked into 360 µL of human whole blood. 20 µL of internal standard spiking solution was added, along with 1.14 mL of cold acetonitrile. The samples were vortexed for 30 sec and centrifuged for 5 min at 8,000 rpm. 500 µL of supernatant was transferred to a clean tube, dried down under N_2, and reconstituted using 125 µL of mobile phase A. The subsequent sample was centrifuged for 5 min at 8,000 rpm and the supernatant was transferred to a LC vial for analysis.

HPLC Conditions:
HPLC separation was performed at 30ºC using a Phenomenex C18 column on the SCIEX ExionLC™ AC system. Mobile phase consisted of water, methanol, acetonitrile and modifiers. The LC flow rate was 0.3 mL/min and the total run time was 17 min.

Mass Spectrometry:
Data was collected using positive electrospray ionization on the QTRAP® 4500 system with Analyst® Software 1.7. *Scheduled* MRM™ algorithm was used to collect the appropriate amount of data points for quantifiable data.

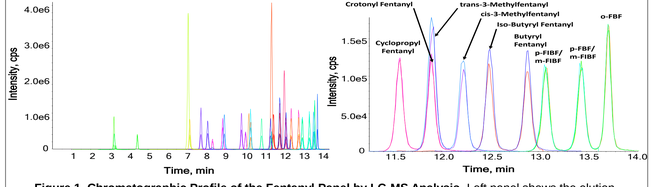

Figure 1. Chromatographic Profile of the Fentanyl Panel by LC-MS Analysis. Left panel shows the elution of 34 compounds in a 17-minute runtime. Right panel highlights the baseline separation achieved on 9 isomers from 11.3 to 14.0 minutes.

RESULTS

Figure 1 displays XIC chromatograms of the fentanyl panel by LS-MS analysis. Nine fentanyl analogues (trans-3-MethylFentanyl, cis-3-Methylfentanyl, Iso-Butyryl Fentanyl, Butyryl Fentanyl, p-FIBF/m-FIBF, p-FBF/m-FBF, o-FBF, Cyclopropyl Fentanyl, Crotonyl Fentanyl) were isomeric with other analogues on panel and thus have no unique fragments that could be used for detection. Therefore, chromatographic separation of these analogues from their isomers is critical for confident identification and quantitation and therefore was the focus of this study.

Optimal chromatographic separation was accomplished by using a Phenomenex C18 column which allowed for better retention and selectivity of the more polar analytes throughout the gradient. The column in conjunction with an optimized mobile phase composition produced the separation that was needed to correctly distinguish all isomers (excluding p-FBF/m-FBF and p-FIBF/m-FIBF). This chromatographic separation was optimized for this MRM assay but can also be used on the SCIEX X500R QTOF, for additional screening or confirmation techniques.

Figure 2 shows the XIC chromatograms of specific fentanyl isomers. Analogues p-FBF and m-FBF, as well as p-FIBF and m-FIBF, were unable to be separated chromatographically so they were combined to be analyzed together as p-FBF/m-FBF and p-FIBF/m-FIBF. This is often the case with chemically similar positional isomers, as there is a trade-off between separation of ortho-, meta-, and para- species and desirable analysis time. For purposes of this study, it was determined that identification as a set paired analogues was the best strategy.

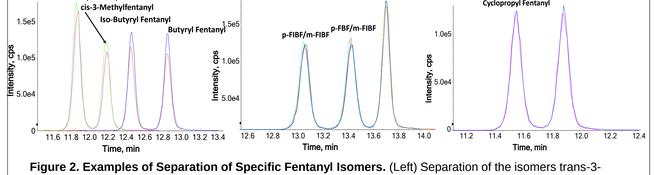

Figure 2. Examples of Separation of Specific Fentanyl Isomers. (Left) Separation of the isomers trans-3-MethylFentanyl, cis-3-Methylfentanyl, Iso-Butyryl Fentanyl, Butyryl Fentanyl from 11.3 to 12.1 minutes. (Middle) Separation of the isomers p-FIBF/m-FIBF, p-FBF/m-FBF, o-FBF from 12.8 to 14.0 minutes. (Right) Separation of the isomers Cyclopropyl Fentanyl, Crotonyl Fentanyl from 11.3 to 12.1 minutes.

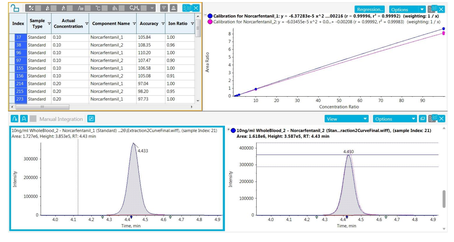

Figure 3. Data Processing of Norcarfentanil using SCIEX OS Software 1.4 (Top) Results table with calibration curve from 0.1 ng/mL to 100 ng/mL. (Bottom) First and second transition with ion ratio overlay.

A processing method for the 29 analytes and 5 internal standards was used to review the time-scheduled MRM data in SCIEX OS Software 1.4. Figure 3 displays the data review of Norcarfentanyl and include the results table with calibration curve along with the first and second transition with ion ratio overlay. Utilizing the MQ4 algorithm in Analytics, detection and integration of the peaks from the background was easily accomplished. The MQ4 algorithm provided selection of the correct peak within the viewing window, especially in the case of the isomers where more than one peak were present. This ease of use helped in analyzing replicate injections (n=3) which were used to verify the consistency of ion ratios and to confirm the accuracy of concentration values within ± 20%, as shown in Figure 3 and 4.

With the whole blood samples being diluted by a factor of 10 and a low injection volume of 5 µL, all analytes were successfully able to be detected in matrix at 0.1 ng/mL. The least sensitive analyte was norfentanyl with a S/N ratio of 51.8 for the qualifier transition.

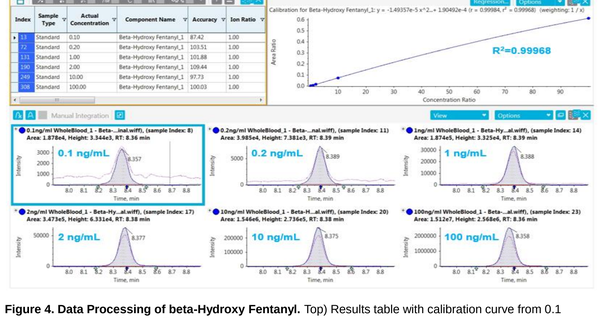

Figure 4. Data Processing of beta-Hydroxy Fentanyl. Top) Results table with calibration curve from 0.1 ng/mL to 100 ng/mL (Bottom) First and second transition with ion ratio overlay.

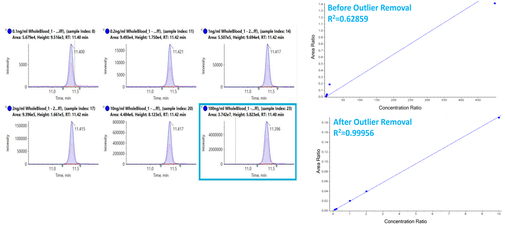

Figure 5. Automatic Outlier Rejection Improves Data Quality. (Left) MRM traces for 2-Furanyl Fentanyl across the concentration range. (Top Right) Concentration curve across all 6 concentrations, showing saturation at the highest concentration, resulting in poor linear fit. (Bottom Right) Automated outlier rejection removed the top concentration which provided a much better linear fit for the concentration curve.

A new feature in the Analytics section of SCIEX OS Software 1.4 is automatic outlier removal. This new feature will remove outliers based on criteria set by the user. In the example below, a highly concentrated calibrator was purposely injected to cause saturation on the high end of the calibration range (Figure 5). The automatic outlier algorithm was easily applied, removing the highly concentrated sample from the calibration curve and improving the fit and correlation of the concentration curve (Figure 5, bottom right).

CONCLUSIONS

The QTRAP 4500 system combined with the ExionLC AC system enabled the separation of fentanyl, its analogues, and metabolites, while maintaining the sensitivity at low concentrations in complex biological matrices such as human whole blood. The *Scheduled* MRM algorithm in Analyst Software 1.7 and the speed of the QTRAP 4500 system produced ample acquisition points for quality data that is easily quantifiable.

- Baseline separation of fentanyl analogues and isomers.
- LC chromatographic method can be used on other platforms, including the SCIEX X500R QTOF, for screening and confirmatory techniques.
- Dynamic range for quantitation averaged ~4 orders of magnitude across the compounds monitored in this study.

REFERENCES

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TRADEMARKS/LICENSING

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