LC/MS/MS Analysis of Fentanyl and Related Analogs Using Biocompatible Solid Phase Microextraction

Petra Lewits[1], Emily Barrey[2], Cory E. Muraco[3]

Merck KGaA Darmstadt, Germany

[1]Applied Solutions Advanced Analytical, Frankfurter Strasse 250, 64239 Darmstadt, Germany, Global Product Manager HPLC columns
[2]Sample Preparation and Workflow R&D, Bellefonte PA USA, Senior R&D Scientist
[3]Liquid Separations R&D, Bellefonte PA USA, Senior R&D Scientist

Contact: petra.Lewits@merckgroup.com

Introduction

Fentanyl is a controlled substance and has been categorized as a Schedule II drug under the "Controlled Substance Act" in the United States. Fentanyl and related compounds are synthetic opioids that are at least 100 times more potent than morphine. Their main therapeutic applications are intravenous or intramuscular analgesia and sedation and have been widely used for neuroleptic analgesia and surgical anesthesia at does ranging from 2 – 50 g/mL.
However, the past five years have seen a significant increase in the trafficking and usage of synthetic opioids with a preference for fentanyl. Due to the highly addictive nature of fentanyl and its analogues, several communities worldwide are experiencing an epidemic of opioid-induced overdoses, criminal activity, and lost productivity. In addition to abuse of prescribed fentanyl and other opioids, many "underground" drug laboratories are synthesizing illicit analogues of fentanyl, such as acetyl fentanyl and butyryl fentanyl, which have been designed to evade screening and prosecution by drug enforcement agencies. As the number of opioid drugs and deaths increases, there is a growing need for analytical methods to quickly and accurately determine the concentrations of these drugs in biological samples.

Experimental

In this study, fentanyl and related analogues were extracted from urine using a mixed mode (C8/strong cation exchanger (SCX)) BioSPME fiber and subsequently analyzed using an Ascentis Express Biphenyl column. The structures of fentanyl and its related analogs are:

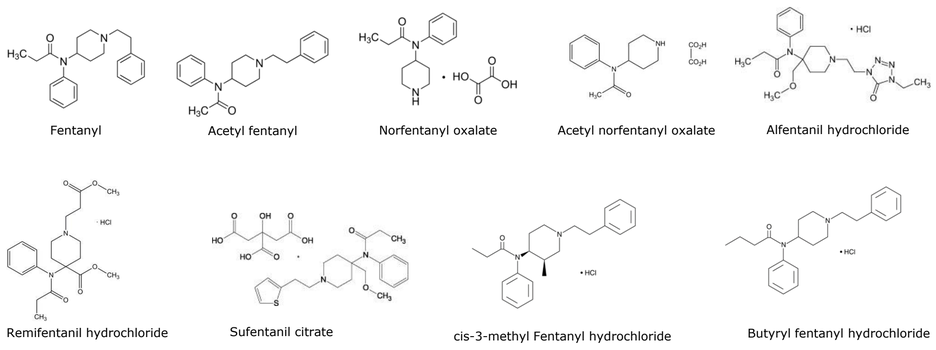


Figure 1: structures of fentanyl and its related analogs

Examination of the structures of these compounds reveals that all these compounds have several sets of delocalized pi electrons either through the benzene ring or centered around the amide functional group. The Ascentis Express Biphenyl column incorporates ligands with biphenyl moieties which are also rich in pi electrons. Therefore, pi-pi stacking can occur between the analytes and the stationary phase, enabling unique interaction between the compounds and the stationary phase. In addition, the planar structure of the biphenyl ligand enables the column to discriminate structurally similar analytes, allowing for increased resolution between structurally similar compounds.

Extraction

A spiked urine sample was subjected to extraction with a mixed mode BioSPME fiber. The fiber was conditioned in 50:50 methanol:water (1 mL, 30 min, 800 rpm agitation). The fiber was rinsed off with water (1 mL, 10 s, 800 rpm) prior to extraction. The fiber was immersed into the urine sample and extraction could proceed (1 mL, 30 min, 800 rpm) followed by a water rinse (1 mL, 10 s, 800 rpm). The analytes were desorbed from the fiber using 90:10 methanol:water containing 0.1% (v/v) ammonium hydroxide (200 µL, 30 min, 800 rpm agitation).

Mixed mode (C8/SCX) fibers were conditioned in 1 mL of 50:50 (methanol/water) for 30 min with an agitation rate of 800 rpm. Followed by a water rinse for ~ 10 s at 800 rpm.

↓

Fibers were placed into 1 mL spiked urine samples and extracted for 30 min at 800 rpm, followed by a water rinse for ~ 10 s at 800 rpm.

↓

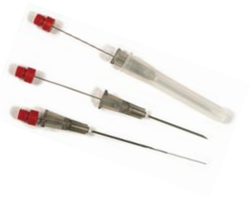
Fibers were desorbed in 0.1% ammonium hydroxide in 90:10 methanol:water for 10 min at 800 rpm

↓

Samples then analyzed by LC/MS/MS

BioSPME

Biocompatible solid phase microextraction (BioSPME) is a variant of solid phase microextraction (SPME) in which the SPME fibers are coated with a non-swelling, biocompatible polymer. The benefit of this design enables minimized binding of biomacromolecules such as proteins and phospholipids but allows extraction of smaller analytes of interest. This coating enables the end user to directly extract analytes out of complex matrices without risk of proteins interfering with downstream quantitation of the analytes of interest. Using BioSPME eliminates many steps found in SPE methods and eliminates matrix effects often seen with dilute and shoot approaches.



The BioSPME technique only extracts the free portion of a drug within a biological sample; therefore, before sample quantitation can occur, a series of extracted standard curves were prepared for each analyte. These calibration samples, which were spiked in synthetic urine, were used to determine the average recovery of each analyte within the spiked samples.

LC/MS/MS Analysis of Fentanyl and Related Compounds

Due to the aromatic nature of the analytes of interest, an Ascentis Express Biphenyl column was employed for the separation of the nine fentanyl analogs. The chromatogram below shows the LC/MS/MS results of the analysis. The Ascentis Express Biphenyl column provided good resolution of the nine fentanyl analogs which allowed for accurate quantitation of the analytes.

Column: **Ascentis Express Biphenyl**, 5 cm x 2.1 mm, 2.7 µm
Mobile phase
A: 0.1% formic acid in water
B: 0.1% formic acid in methanol
Gradient: 40%B to 50%B in 2 min, to 80%B in 1 min, hold 80%B for 1 min, to 40%B in 0.1 min and hold at 40%B for 1.4 min
Flow rate: 0.6 mL/min
Column temp: 50 °C
Det.: MS/MS, ESI (+), MRM
Injection: 4 µL
Instrument: Agilent® 1290 Infinity II with 6460 QQQ

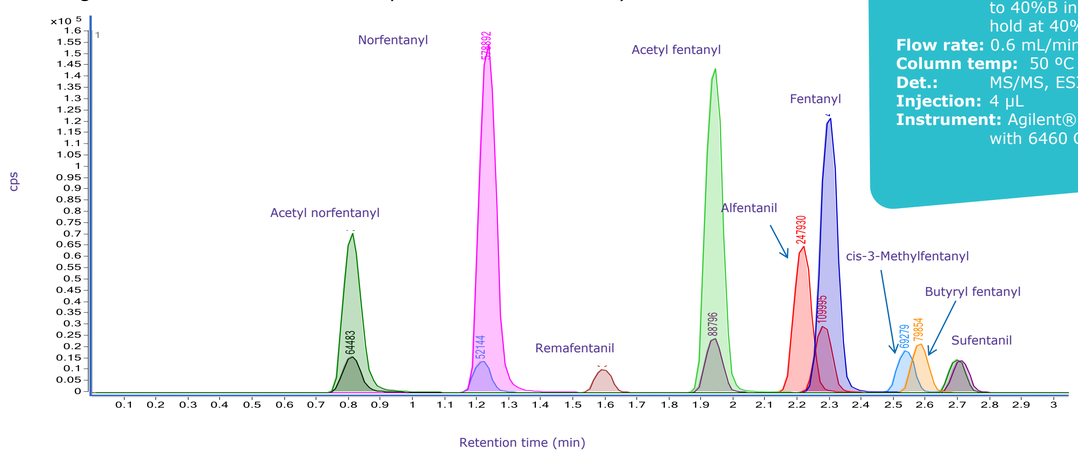


Figure 2: LC/MS/MS results of the analysis of the nine fentanyl analogs on Ascentis Express Biphenyl column

Quantification and Recovery

The table below outlines the quantitation results of the experiment for each analyte. Recoveries for each analyte ranged from 66.7% to 111%. All the analytes had recoveries greater than 70% at 0.05 ng/mL except for remafentanil and alfentanil. However, the lower recoveries may be an artifact of the experiment: stable label internal standards were not available for remafentanil and alfentanil. Having a matched internal standard for these two compounds would have improved the calculated recovery. Examination of the precision of the method also revealed a high degree of reproducibility as the percent relative standard deviation (%RSD) of the method was less than 10% for most analytes except for remafentanil and alfentanil. Again, the lower degree of precision for these two compounds could be due to the lack of an exact match internal standard.

	0.05 ng/mL		0.1 ng/mL		1 ng/mL	
Compound	Avg. % Rec	%RSD	Avg. % Rec	%RSD	Avg. % Rec	%RSD
Acetyl norfentanyl	83.9	7.6	90.1	6.1	98.5	1.6
Norfentanyl	78.5	2.5	86.1	2.9	99.2	0.8
Remafentanil*	BLQ	-	BLQ	-	111	18.9
Acetyl fentanyl	86.4	4.6	87.3	2.5	93.4	1.9
Alfentanil^	BLQ	-	66.7	14.7	78.2	10.6
Fentanyl	90.4	6.3	87.5	2.6	91.1	1.1
Butyryl fentanyl^	83.9	2.1	76.9	4.2	76	5.7
cis-3-methylfentanyl^	93.9	5.1	84.6	5.6	84	4.6
Sufentanil	95.3	6.1	88.6	4.9	84.8	2.6

BLQ = Below limit of quantitation
* used Acetyl fentanyl-13C as internal standard
^ used fentanyl-d5 as internal standard

Table 1: Quantitation results of the analysis of the nine fentanyl analogs

Besides the matrix's affinity for analytes of interest in a biological sample, there is also the possibility of matrix components interacting with the extraction adsorbent, thus skewing the results of the quantitation to lower values. This observation is due to biomacromolecules in the sample binding to interaction sites on the adsorbent thereby preventing analytes from being able to sample all the available surface area of the adsorbent. The BioSPME coating, which contains functionalized C18 particles and a proprietary binder, has been developed to prevent non-specific binding of biomacromolecules to the coating. To test this coating, the effect the matrix had on the analyte response was evaluated by spiking analyte into extracted urine blank samples and comparing the response to analyte spiked into the desorption solution. The Figure below shows the results of this experiment by comparing the "percent matrix effects" for each compound. The percent matrix effects are calculated by taking a ratio of the analyte response in the presence of matrix to the analyte response in the absence of matrix. As can be seen, the percent matrix effects were calculated to be less than 10% for all the analytes indicating that the extraction protocol has eliminated most of the impact of the matrix on the analysis

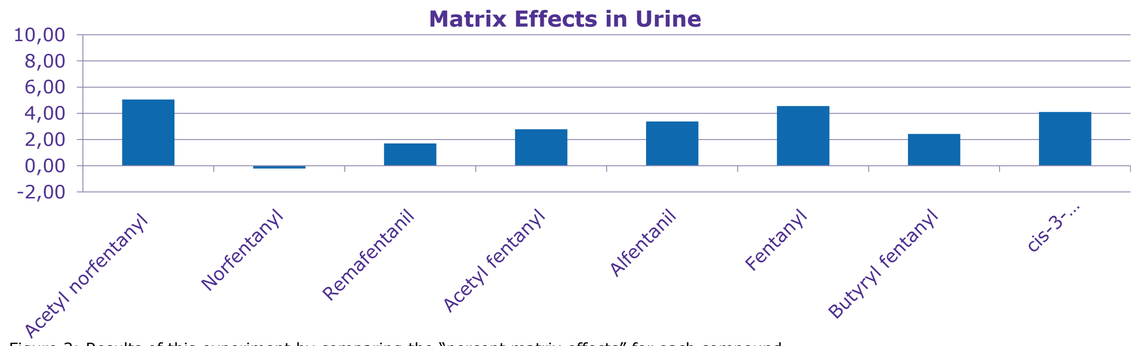


Figure 3: Results of this experiment by comparing the "percent matrix effects" for each compound

Conclusion

The rise and spread of opioid abuse, especially fentanyl, is a growing concern in the healthcare industry. To better detect fentanyl and related analogs, a simple, three step extraction utilizing BioSPME fiber tips was developed for fast, reproducible detection. Linear responses from 0.05 ng/mL to 50 ng/mL were established for all analytes except for remafentanil which displayed a linear response from 1 ng/mL to 10 ng/mL. Limits of quantitation (LOQ) were demonstrated at 0.05 ng/mL for most compounds, except for remafentanil and alfentanil, which were at 1 ng/mL. The use of an Ascentis Express Biphenyl Fused-Core column yielded excellent separation of all nine analytes in less than three minutes with an overall run time of 5.5 min. Finally, due to the biocompatible coating of the adsorbent, constituents of the matrix did not interfere with analyte quantitation as matrix effects resulted in a less than 10% deviation between solvent-rich and matrix-rich samples. Combining the simple microextraction technique with the fast LC/MS/MS method, sub-ng/mL detection limits are possible for high throughput analysis of urine samples using BioSPME.

Lit. No. MK_PS4397EN 07/2019

Supelco®
Analytical Products