Analysis of Fentanyl and Its Analogues in Human Urine by LC-MS/MS

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Abstract

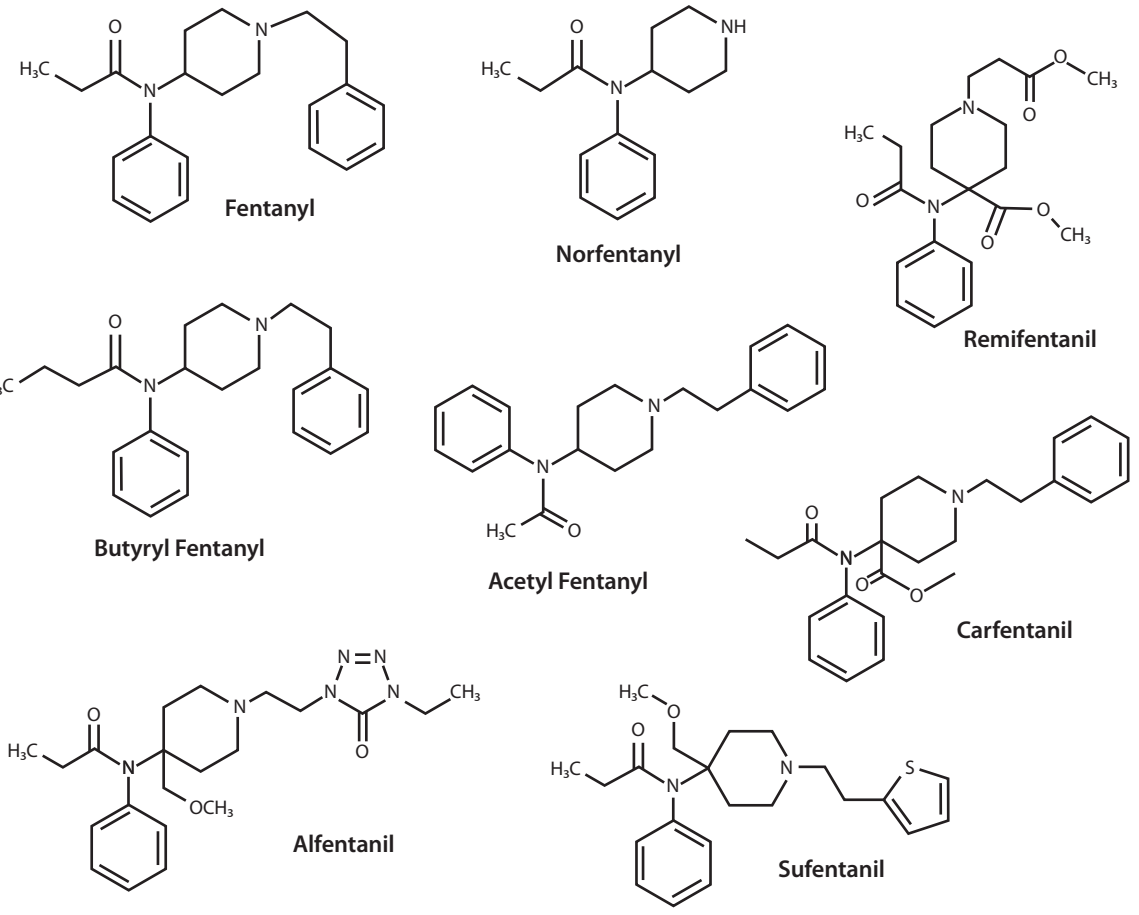
Synthetic opioid drugs, such as fentanyl and sufentanil, have very high analgesic potency. Abuse of these prescription painkillers—along with a rapidly growing list of illicit analogues—is a significant public health problem. In this study, we developed a simple dilute-and-shoot method that provides a fast 3.5 minute analysis of fentanyl and related compounds (norfentanyl, acetyl fentanyl, alfentanil, butyryl fentanyl, carfentanil, remifentanil, and sufentanil) in human urine by LC-MS/MS using a Raptor Biphenyl column.

Introduction

In recent years, the illicit use of synthetic opioids has skyrocketed, and communities worldwide are now dealing with an ongoing epidemic. Of the thousands of synthetic opioid overdose deaths per year, most are related to fentanyl and its analogues. With their very high analgesic properties, synthetic opioid drugs such as fentanyl, alfentanil, remifentanil, and sufentanil are potent painkillers that have valid medical applications; however, they are also extremely addictive and are targets for abuse. For example, carfentanil is a very powerful anesthetic used as a tranquilizer for large animals, primarily elephants. It is 10,000 times more potent than morphine, making it one of the most powerful synthetic opioids available. The increase in its illicit use, most commonly by mixing with heroin, has been linked to a significant number of overdose deaths since 2016. In addition to abuse of these prescription drugs, the current opioid crisis is fueled by a growing number of illicit analogues, such as acetyl fentanyl and butyryl fentanyl, which have been designed specifically to evade prosecution by drug enforcement agencies.

As the number of opioid drugs and deaths increases, so does the need for a fast, accurate method for the simultaneous analysis of fentanyl and its analogues. Therefore, we developed this LC-MS/MS method for measuring fentanyl, six analogues, and one metabolite (norfentanyl) in human urine (Figure 1). A simple dilute-and-shoot sample preparation procedure was coupled with a fast (3.5 minutes) chromatographic analysis using a Raptor Biphenyl column. This method provides accurate, precise identification and quantitation of fentanyl and related compounds, making it suitable for a variety of testing applications including clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

Figure 1: Chemical Structures



Experimental

Sample Preparation

The analytes were fortified into pooled human urine. An 80 µL urine aliquot was mixed with 320 µL of 70:30 water:methanol solution (five-fold dilution) and 10 µL of internal standard (40 ng/mL in methanol) in a Thomson SINGLE StEP filter vial (Restek cat.# 25895). After filtering through the 0.2 µm PVDF membrane, 5 µL was injected into the LC-MS/MS.

Calibration Standards and Quality Control Samples

The calibration standards were prepared in pooled human urine at 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/mL. Three levels of QC samples (0.75, 4.0, and 20 ng/mL) were prepared in urine for testing accuracy and precision with established calibration standard curves. Recovery analyses were performed on three different days. All standards and QC samples were subjected to the sample preparation procedure described above.

LC-MS/MS analysis of fentanyl and its analogues was performed on an ACQUITY UPLC instrument coupled with a Waters Xevo TQ-S mass spectrometer. Instrument conditions were as follows, and analyte transitions are provided in Table I.

Analytical column:	Raptor Biphenyl (5 µm, 50 mm x 2.1 mm; cat.# 9309552)
Guard column:	Raptor Biphenyl EXP guard column cartridge, (5 µm, 5 mm x 2.1 mm; cat.# 930950252)
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in methanol

Gradient	Time (min)	%B
	0.00	30
	2.50	70
	2.51	30
	3.50	30

Flow rate:	0.4 mL/min
Injection volume:	5 µL
Column temp.:	40 °C
Ion mode:	Positive ESI

Table I: Ion Transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier	Internal Standard
Norfentanyl	233.27	84.15	56.06	Norfentanyl-D_5
Acetyl fentanyl	323.37	188.25	105.15	Acetyl fentanyl-$^{13}C_6$
Fentanyl	337.37	188.26	105.08	Fentanyl-D_5
Butyryl fentanyl	351.43	188.20	105.15	Carfentanil-D_5
Remifentanil	377.37	113.15	317.30	Norfentanyl-D_5
Sufentanil	387.40	238.19	111.06	Sufentanil-D_5
Carfentanil	395.40	113.14	335.35	Carfentanil-D_5
Alfentanil	417.47	268.31	197.23	Acetyl fentanyl-$^{13}C_6$
Norfentanyl-D_5	238.30	84.15	—	—
Acetyl fentanyl-$^{13}C_6$	329.37	188.25	—	—
Fentanyl-D_5	342.47	188.27	—	—
Sufentanil-D_5	392.40	238.25	—	—
Carfentanil-D_5	400.40	340.41	—	—

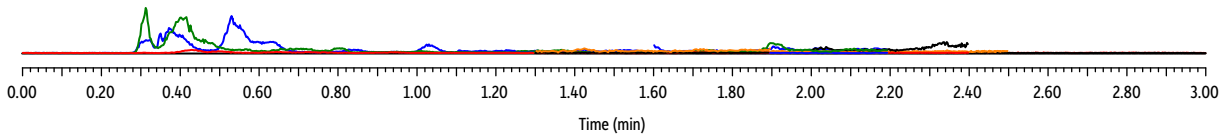
Results and Discussion

Chromatographic Performance

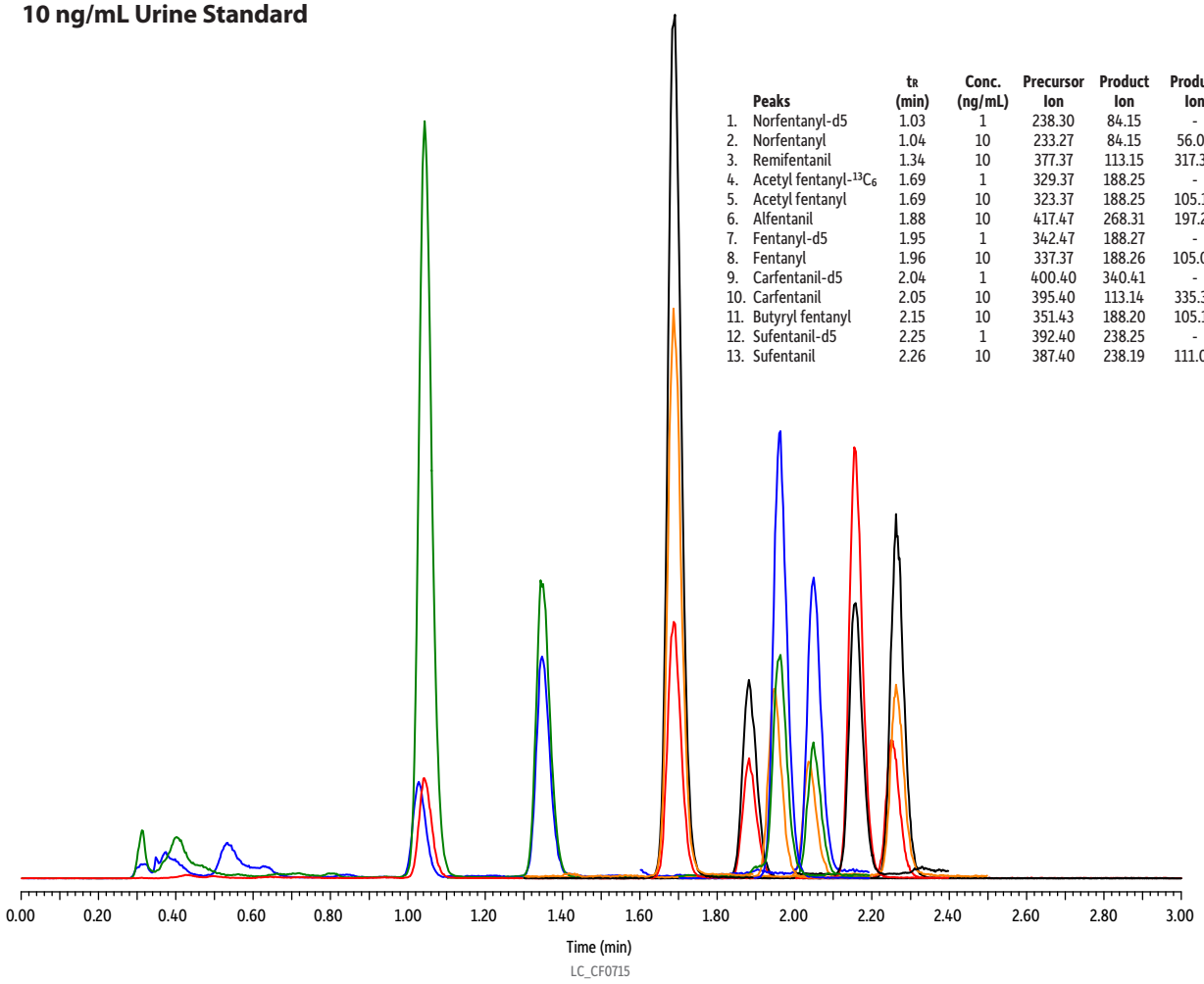
All eight analytes were well separated within a 2.5-minute gradient elution (3.5-minute total analysis time) on a Raptor Biphenyl column (Figure 2). No significant matrix interference was observed to negatively affect quantification of the five-fold diluted urine samples. The 5 µm particle Raptor Biphenyl column used here is a superficially porous particle (SPP) column. It was selected for this method in part because it provides similar performance to a smaller particle size fully porous particle (FPP) column, but it generates less system backpressure.

Figure 2: The Raptor Biphenyl column effectively separated all target compounds in urine with no observed matrix interferences.

Blank Urine



10 ng/mL Urine Standard



	Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion	Product Ion
1.	Norfentanyl-d5	1.03	1	238.30	84.15	-
2.	Norfentanyl	1.04	10	233.27	84.15	56.06
3.	Remifentanil	1.34	10	377.37	113.15	317.30
4.	Acetyl fentanyl-$^{13}C_6$	1.69	1	329.37	188.25	-
5.	Acetyl fentanyl	1.69	10	323.37	188.25	105.15
6.	Alfentanil	1.88	10	417.47	268.31	197.23
7.	Fentanyl-d5	1.95	1	342.47	188.27	-
8.	Fentanyl	1.96	10	337.37	188.26	105.08
9.	Carfentanil-d5	2.04	1	400.40	340.41	-
10.	Carfentanil	2.05	10	395.40	113.14	335.35
11.	Butyryl fentanyl	2.15	10	351.43	188.20	105.15
12.	Sufentanil-d5	2.25	1	392.40	238.25	-
13.	Sufentanil	2.26	10	387.40	238.19	111.06

Column Raptor Biphenyl (cat.# 9309552)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 5 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard cartridge 5 mm, 2.1 mm ID, 5 µm (cat.# 930950252)
Temp.: 40 °C
Sample
Diluent: 70:30 Water:methanol
Inj. Vol.: 5 µL
Mobile Phase
A: 0.1% Formic acid in water
B: 0.1% Formic acid in methanol

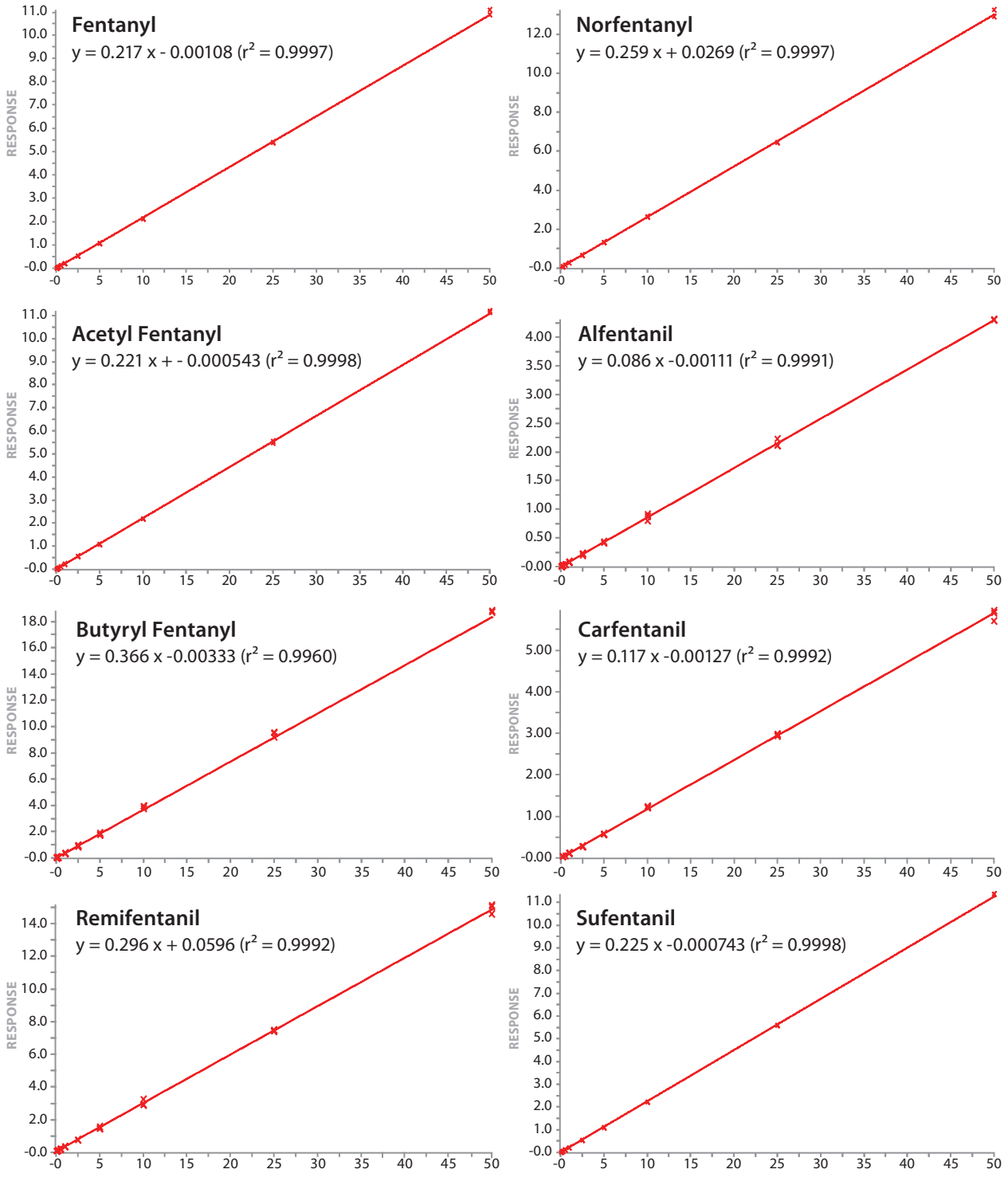
Time (min)	Flow (mL/min)	%A	%B
0.00	0.4	70	30
2.50	0.4	30	70
2.51	0.4	70	30
3.50	0.4	70	30

Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument UHPLC
Notes Human urine was fortified at 10 ng/mL with target analytes. An 80 µL urine aliquot was mixed with 320 µL of 70:30 water:methanol solution (5-fold dilution) and 10 µL of internal standard solution (40 ng/mL in methanol) in a Thomson SINGLE StEP filter vial (Restek cat.# 25895). After filtering through the 0.2 µm PVDF membrane, 5 µL was injected for analysis.

Linearity

Linear responses were obtained for all compounds and the calibration ranges encompassed typical concentration levels monitored for both research and abuse. Using 1/x weighted linear regression ($1/x^2$ for butyryl fentanyl), calibration linearity ranged from 0.05 to 50 ng/mL for fentanyl, alfentanil, acetyl fentanyl, butyryl fentanyl, and sufentanil; from 0.10 to 50 ng/mL for remifentanil; and from 0.25 to 50 ng/mL for norfentanyl and carfentanil. All analytes showed acceptable linearity with r^2 values of 0.996 or greater (Figure 3) and deviations of <12% (<20% for the lowest concentrated standard).

Figure 3: Calibration Curves



Accuracy and Precision

Based on three independent experiments conducted on multiple days, method accuracy for the analysis of fentanyl and its analogues was demonstrated by the %recovery values, which were within 10% of the nominal concentration for all compounds at all QC levels. The %RSD range was 0.5-8.3% and 3.4-8.4% for intraday and interday comparisons, respectively, indicating acceptable method precision (Table II).

Table II: Accuracy and Precision Results for Fentanyl and Related Compounds in Urine QC Samples.

	QC Level 1 (0.750 ng/mL)			QC Level 2 (4.00 ng/mL)			QC Level 3 (20.0 ng/mL)		
Analyte	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD
Acetyl fentanyl	0.761	102	1.54	3.99	99.7	2.08	19.9	99.3	0.856
Alfentanil	0.733	97.6	3.34	3.96	98.9	8.38	20.9	104	6.73
Butyryl fentanyl	0.741	98.9	6.29	3.77	94.3	6.01	20.8	104	4.95
Carfentanil	0.757	101	7.34	3.76	94.0	4.64	20.6	103	4.24
Fentanyl	0.761	102	1.98	3.96	99.1	2.31	19.9	99.6	1.04
Norfentanyl	0.768	103	6.50	4.04	101	1.84	20.1	101	2.55
Remifentanil	0.765	102	3.42	3.97	99.2	3.68	20.8	104	4.14
Sufentanil	0.752	100	1.67	3.93	98.3	1.28	20.1	100	0.943

Conclusion

A simple dilute-and-shoot method was developed for the quantitative analysis of fentanyl and its analogues in human urine. The analytical method was demonstrated to be fast, rugged, and sensitive with acceptable accuracy and precision for urine sample analysis. The Raptor Biphenyl column is well suited for the analysis of these synthetic opioid compounds and this method can be applied to clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

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