Article

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Quantitative Measurement of Acetyl Fentanyl and Acetyl Norfentanyl in Human Urine by LC-MS/MS

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Keywords: Acetyl fentanyl, emerging drug of abuse**,** human testing**,** human metabolism

ABSTRACT

Opioid abuse involving emerging opioid compounds is a growing public health problem, which was highlighted recently by cases of human morbidity and mortality linked to acetyl fentanyl abuse. Unfortunately, the lack of information available on the toxicology and metabolism of acetyl fentanyl precludes its detection in human samples. The following study was conducted to test a new analytical procedure for the simultaneous quantification of acetyl fentanyl and its predicted metabolite, acetyl norfentanyl, in human urine. Metabolic reference standards and deuterium-labeled internal standards were synthesized for use in an assay that coupled solid-phase extraction (SPE) with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The accuracy (% Relative Error <5%), inter- and intra-run precision (%CV <20%) of this new method resulted in low levels of quantification (~ 1 ng/ml). Similar results were obtained using liquid chromatography columns manufactured with phenyl-hexyl and biphenyl stationary phases (r^2 > 0.98). Preliminary human liver microsomal and in vivo rodent studies demonstrated that acetyl fentanyl is metabolized by cytochrome P450 to acetyl norfentanyl. Urine samples from rats treated with a toxic dose of acetyl fentanyl contained high concentrations of acetyl fentanyl and acetyl norfentanyl. Further toxicokinetic studies are required to fully elucidate the metabolic pathways responsible for acetyl fentanyl detoxification and excretion.

INTRODUCTION

Abuse of prescription opioids is an ongoing epidemic that continues to burden the public health system. Since 2001, opioid abuse has been of great concern and 12.2% of high school seniors report use of prescription narcotics for non-medical reasons.[1,2] In addition, unintentional poisoning deaths involving opioid analgesics have increased over the last decade.[3,4] For example, deaths from opioid pain relievers increased five-fold in women between 1999 and 2010, and since 1997, opioid-related drug overdoses exceeded motor vehicle collisions as the cause of death in this population.[5] The etiology of increased opioid-related deaths is multifactorial and includes ready-to-use heroin and fentanyl tablet formulations[6,7] and synthetic opioid derivatives that circumvent analog drug laws and detection in routine toxicological assays. For example, United States' analog laws were implemented in response to "China White" (4-methylfentanyl) as early as the 1980s[9], but now acetyl fentanyl is the 'new emerging' drug of abuse that escapes detection and is linked to over 50 human fatalities in Rhode Island and Pennsylvania.[11-13] Acetyl fentanyl has never been approved for human use and is considered a controlled substance analogue.[13]

When faced with an unsuspected acetyl fentanyl death, forensic toxicology laboratories may be perplexed when enzyme-linked immunosorbent assay (ELISA) screens are positive for fentanyl, yet confirmatory gas chromatography-mass spectrometry (GC-MS) and/or liquid chromatography-mass spectrometry (LC-MS) analyses yield no fentanyl result. Thus, development of a validated testing procedure to quantify acetyl fentanyl and acetyl norfentanyl in human samples is critical to accurately

diagnosing human morbidity and mortality from this deadly emerging drug of abuse. This study reports a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method for acetyl fentanyl and the predicted human metabolite acetyl norfentanyl.

EXPERIMENTAL SECTION

Reagents and Chemicals

Acetyl fentanyl, acetyl norfentanyl, acetyl fentanyl-d_5, and acetyl norfentanyl-d_5 standards were provided by Cayman Chemical (Ann Arbor, MI). Sodium acetate trihydrate, ammonium formate, and β-glucuronidase were purchased from Sigma-Aldrich (St. Louis, MO). Solid phase extraction was performed using Strata X-Drug-B polymeric strong cation-exchange cartridges provided by Phenomenex (Torrance, CA). Pooled human urine void of synthetic opioids is maintained by the Arkansas Public Health Laboratory and routinely used as a drug-free blank sample for various clinical analyses. Deionized water was purified to resistivity of 18.2 mΩ-cm using an Elga Purelab Ultra laboratory water purification system (Woodridge, IL). Optima grade acetonitrile and other analytical grade chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

Equipment

Aliquots of all specimens and standards were extracted using solid phase extraction (SPE) on a Gilson Nebula 215 solid-phase extraction system (Middleton, WI) controlled by Gilson 735 Sampler software. Extracted samples were analyzed using an Agilent 1200 series quaternary liquid chromatography system (Santa Clara, CA) interfaced with an API-4000 Q-Trap tandem mass spectrometer (AB SCIEX, Framingham, MA). The operation of the LC-MS/MS system was controlled by Analyst

software (version 1.5.1, AB SCIEX, Framingham, MA).

Preparation of Analytical Standards and Quality Control Material

Analytical calibration standards and quality control (QC) material were prepared from a common aqueous stock solution containing 10 µg/mL of acetyl fentanyl and acetyl norfentanyl. An internal standard spiking solution (100 ng/ml final concentration of each analyte) was prepared in deionized water from a common aqueous stock solution containing 10 µg/mL of acetyl fentanyl-d_5 and acetyl norfentanyl-d_5. All analytical and internal standard solutions were stored at -40°C until needed.

All calibration standards and QCs were matrix matched by first preparing a 500 ng/mL intermediate working standard in pooled, blank human urine. Final working urine standards (0 ng/mL to 100 ng/mL) were prepared daily by serially diluting the intermediate working standard with blank urine. QCs were prepared in urine independent of calibration standards and at three concentrations spanning the calibration range (QCL, QCM, and QCH).

β-Glucuronidase Treatment & Solid Phase Extraction

Sample hydrolysis procedures were incorporated as part of this new analytical method because several synthetic opioids like morphine are conjugated with glucuronic acid prior to urinary excretion.[16] Thus, it is important to validate testing procedures to ensure they are capable of using hydrolyzed urine samples. All standards, QCs, and samples were subjected to β-glucuronidase treatment prior to solid-phase extraction

(SPE). Urine standards, QCs, and samples were prepared identically by diluting 80 µL aliquots with 310 µL of 0.1 M sodium acetate buffer (pH 5.0) containing β-glucuronidase, spiking with 10 µL of the internal standard solution, and incubating for 60 minutes at 37°C.

The entire reaction mixture (400 µL) was loaded onto the polymeric strong-cationic exchange solid phase extraction cartridge (Strata X-B 33 µ, 30 mg/3mL), washed with 1 mL of a 0.1% formic acid aqueous solution and 1 mL of a 70:30 water:methanol solution. Analytes were eluted twice with 0.5 mL of a basic 50:50 methanol:acetonitrile solution (2% ammonium hydroxide). The collected eluent was evaporated at 60°C under a stream of nitrogen until dry, and analytes were reconstituted in a 50:50 mixture of mobile phase (85% mobile phase A and 15% mobile phase B) and acetonitrile. Mobile phase A (MPA) consisted of 10 mM ammonium formate in water and mobile phase B (MPB) consisted of 0.1% formic acid in methanol.

Liquid Chromatography Tandem Mass Spectrometry

The primary LC-MS/MS method reported here utilizes a Phenomenex phenyl-hexyl (2.6 µm, 100 Å) analytical column (50 mm x 4.6 mm) heated to 30°C. Analytes are resolved using a gradient starting at 98% MPA, ramping to 2% MPA over 4 min, holding for 2 min, and returning to 98% MPA for an additional 2 min. The total run time was 8 min, including the column equilibration period between injections. Second column confirmation studies were completed using a Restek Raptor biphenyl (2.7 µm) analytical column (50 mm x 3.0 mm). This column required a higher flow rate (800 uL/min), but

used the same mobile phases. Analytes of interest were resolved over a 5 minute total run-time, with a gradient starting at 98% MPA, ramping to 2% MPA over 1.5 minutes, holding for 1.5 minutes, and returning to 98% MPA for an additional 2 minutes. Significant carryover was observed with both columns following the evaluation of highly concentrated samples. Carryover was controlled by injecting a series of blanks after each high standard, QC, and unknown sample.

Electrospray ionization in positive-ion mode was used to collect mass spectral data. Source temperature and turbo ion spray voltage were maintained at 650°C and 5,500 V, respectively. Nitrogen gas settings for the source gases GS1 and GS2, curtain gas and collision gas were 50.0 cm/s, 50.0 cm/s, 20.0 cm/s, and “medium”, respectively. Molecular specific parameters for specific reaction monitoring (SRM) experiments are listed in **Table 1**. The SRM-information dependent acquisition (IDA) transition threshold that triggered enhanced product ion (EPI) experiments was set to an intensity of 500 counts per second. Specific EPI parameters are as follows: 4000 Da/S; 800 – 600 Da; declustering potential (DP) set at 60 V; collision energy spread (CES) set at 5 V; and collision energy (CE) set at 35 V.

Acetyl Fentanyl Metabolic Studies

Pooled human liver microsomes (5 µg) from a 50-donor pool were assayed for cytochrome P450 (CYP) activity toward acetyl fentanyl. Substrate (acetyl fentanyl, 10 µM final concentration) was added in ethanol and allowed to dry at ambient temperature. When dry, protein was added in the presence of 0.5 M potassium

phosphate buffer (pH 7.4) and 10% acetonitrile. Reactions were initiated by the addition of NADPH-regenerating system (solution A: 20 mM NADP+, 60 mM glucose-6-phosphate, and 60 mM $MgCl_2$; solution B: 100 U/mL glucose-6-phosphate dehydrogenase), incubated at 37°C for 60 min, and terminated by the addition of an equal volume of ethanol. Before LC-MS/MS analysis, 10 µL of internal standard spiking mix was added, and proteins were precipitated via centrifugation at 20,800 g for 8 minutes. Controls omitting the substrate, protein, and NADPH were included with each assay, and all incubations were performed in triplicate.

In vivo metabolic studies were performed in two adult, male Sprague Dawley rats placed into new cages with grid floors to facilitate collection of urine. After collection of a baseline urine sample (time 0), acetyl fentanyl dissolved in 0.9% physiological saline was infused through an indwelling intra-jugular venous catheter (3 mg/kg), then flushed with approximately 2.0 mL sterile saline. Urine samples were collected after 10, 180, and 360 min.

Statistical Methods

Accuracy and inter-run precision were determined by evaluating QC results from six independent experiments performed over several non-consecutive days. Intra-run precision was calculated by evaluating results from triplicate QCs prepared independently on a single day. Accuracy was calculated as the absolute percent relative error for each of the expected QC concentrations. Replicate measurements at the three QC concentrations (3.75 ng/mL, 7.5 ng/mL, and 75 ng/mL) were used to determine the

analytical precision, calculated as the percent coefficient of variation (%CV), for both within-run and between-run measurements. The limit of detection was defined at less than the lowest calibrator (0.5 ng/mL for acetyl fentanyl and 1 ng/mL for acetyl norfentanyl), and the lower limit of quantitation (LLQ) was calculated as three times the standard deviation of six replicate analyses of the low QC standard. Correlation studies were evaluated using Pearson correlation coefficients (r^2).

RESULTS AND DISCUSSION

Abuse of synthetic opioids is a growing public health problem, and recent clinical morbidity and mortality reports from Rhode Island and Pennsylvania involving acetyl fentanyl are the latest examples to highlight this dangerous trend.[12,17] Very little is known about the toxicology of this emerging drug of abuse, and several research barriers remain. For example, assays that simultaneously measure the parent drug and primary human metabolites are required to facilitate future clinical studies designed to understand the relationship between drug metabolism and clinical symptoms documented after acetyl fentanyl use.

This report coupled solid-phase extraction with an LC-MS/MS approach that incorporated deuterium labeled internal standards to provide adequate sensitivity and precision for measuring acetyl fentanyl and acetyl norfentanyl. ^{1}H NMR confirmation studies showed chemical shifts and coupling constants that were in agreement with chemical structures (**Figure 1**). Mass spectra showed the presence of molecular ions [MH+] and diagnostic fragment ions (**Figures 2 & 3**).

The LC-MS/MS approach achieved baseline separation of acetyl fentanyl and acetyl norfentanyl and resolved potential interfering substances detected in urine. The isobaric interference (labeled as peak “1”) shown in **Figure 4A** co-eluted with acetyl norfentanyl (peak “2”) in preliminary studies. A slower flow rate allowed baseline resolution of the interference but extended the total run time to 8 min (**Figure 4B**). Initial conditions established for the second column confirmation studies allowed for equivalent resolution of the interference (**Figure 4C**). Chromatography of standards,

QCs, and samples remained consistent throughout this study (**Figure 4B&C**) in which deuterium labeled internal standards co-eluted with each analyte of interest and retention times remained constant (± 0.1 minute). Accuracy, precision, and lower limits of quantification (LLQ) for each analyte in urine are presented in **Table 2**. All calibration curves were linear over the tested analytical range, where r^2 values were ≥0.99.

In some instances, second column confirmation studies may be required to meet clinical or forensic method validation requirements. Therefore, the use of phenyl-hexyl and biphenyl stationary phase columns was examined and no significant differences were detected between the two columns. Fifteen blank human urine samples spiked with acetyl fentanyl and acetyl norfentanyl at concentrations spanning the calibration range had comparable values for both compounds (r^2 =0.98 and 0.99, respectively) (**Figure 5**).

Acetyl fentanyl metabolism was also investigated as part of this study. When incubated with pooled human liver microsomes, hepatic cytochrome P450s catalyzed the production of acetyl norfentanyl (**Figure 6A**). These *in vitro* data are in agreement with previous studies investigating fentanyl metabolism[14,15] and suggest the involvement of similar metabolic pathways. Since authentic human urine samples were not available for this research, rodent studies were included to further assess *in vivo* metabolic pathways and the urinary excretion of acetyl fentanyl and the predicted metabolite acetyl norfentanyl. Baseline urine collected at time zero did not contain either the parent drug or the norfentanyl metabolite, and behavioral observations were entirely species-typical. However, immediately following the intravenous administration of acetyl

fentanyl (i.v. 3 mg/kg), one rat became rigid, lost its righting reflex, and did not respond to nociceptive mechanical pressure applied to the hindlimbs, while the second subject exhibited a sedation consistent with that observed with other opioids. Within approximately 5 minutes, the most impaired rat began to exhibit labored breathing, and manual chest compressions were administered to stimulate ventilation, but the rat expired approximately 15 minutes after drug administration. In contrast, the other rat survived the 6-hour observation period, although it remained sedated the entire time. Urine collected at various times points showed a time-dependent excretion of both acetyl fentanyl and acetyl norfentanyl (**Table 3**). A chromatograph from a urine sample taken three hours post-administration from the rat which survived is shown in **Figure 6B**. Chromatographic and mass spectral data are nearly identical to results obtained with human liver microsomes (**Figure 6A**).

CONCLUSIONS

The LC−MS/MS method presented in this study provides low levels of quantification and a high level of accuracy and reproducibility for acetyl fentanyl and acetyl norfentanyl. The sensitivity, accuracy, and precision of this method are adequate for clinical, public health, and forensic applications. This is the first method to study acetyl fentanyl metabolism and to suggest that human cytochrome P450 metabolism leads to the urinary excretion of acetyl norfentanyl. Future clinical investigations and toxicokinetic studies are required to confirm these preliminary findings and to further elucidate the metabolic pathways responsible for acetyl fentanyl detoxification and excretion.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTION

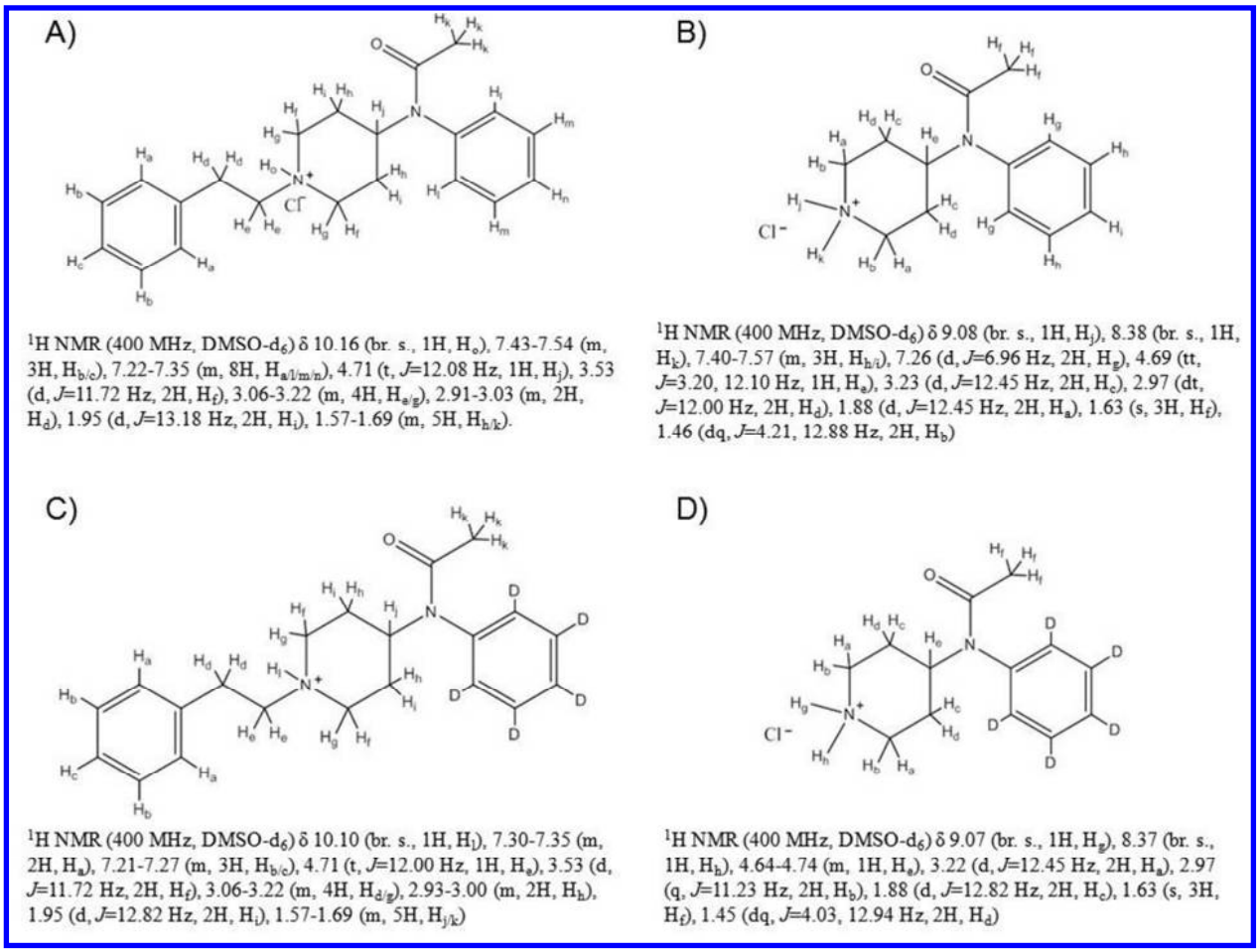
All authors contributed to the writing of this manuscript and the design and/or execution of the studies. All authors provided approval for the final version of this manuscript.

CONFLICT OF INTEREST DISCLOSURE

None of the authors have personal financial conflicts of interest. However Paul Kennedy and Gregory Endres are employees of Cayman Chemical Company. Commercial products from Cayman Chemical Co. were used in the study.

FIGURES

Figure 1.



A) ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.16 (br. s., 1H, H_e), 7.43-7.54 (m, 3H, $H_{b/c}$), 7.22-7.35 (m, 8H, $H_{a/l/m/n}$), 4.71 (t, *J*=12.08 Hz, 1H, H_j), 3.53 (d, *J*=11.72 Hz, 2H, H_f), 3.06-3.22 (m, 4H, $H_{e/g}$), 2.91-3.03 (m, 2H, H_d), 1.95 (d, *J*=13.18 Hz, 2H, H_i), 1.57-1.69 (m, 5H, $H_{h/k}$).

B) ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.08 (br. s., 1H, H_j), 8.38 (br. s., 1H, H_k), 7.40-7.57 (m, 3H, $H_{b/i}$), 7.26 (d, *J*=6.96 Hz, 2H, H_g), 4.69 (tt, *J*=3.20, 12.10 Hz, 1H, H_e), 3.23 (d, *J*=12.45 Hz, 2H, H_c), 2.97 (dt, *J*=12.00 Hz, 2H, H_d), 1.88 (d, *J*=12.45 Hz, 2H, H_a), 1.63 (s, 3H, H_f), 1.46 (dq, *J*=4.21, 12.88 Hz, 2H, H_b)

C) ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.10 (br. s., 1H, H_l), 7.30-7.35 (m, 2H, H_a), 7.21-7.27 (m, 3H, $H_{b/c}$), 4.71 (t, *J*=12.00 Hz, 1H, H_e), 3.53 (d, *J*=11.72 Hz, 2H, H_f), 3.06-3.22 (m, 4H, $H_{d/g}$), 2.93-3.00 (m, 2H, H_h), 1.95 (d, *J*=12.82 Hz, 2H, H_i), 1.57-1.69 (m, 5H, $H_{j/k}$)

D) ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.07 (br. s., 1H, H_g), 8.37 (br. s., 1H, H_h), 4.64-4.74 (m, 1H, H_a), 3.22 (d, *J*=12.45 Hz, 2H, H_a), 2.97 (q, *J*=11.23 Hz, 2H, H_b), 1.88 (d, *J*=12.82 Hz, 2H, H_c), 1.63 (s, 3H, H_f), 1.45 (dq, *J*=4.03, 12.94 Hz, 2H, H_d)

Figure 2.

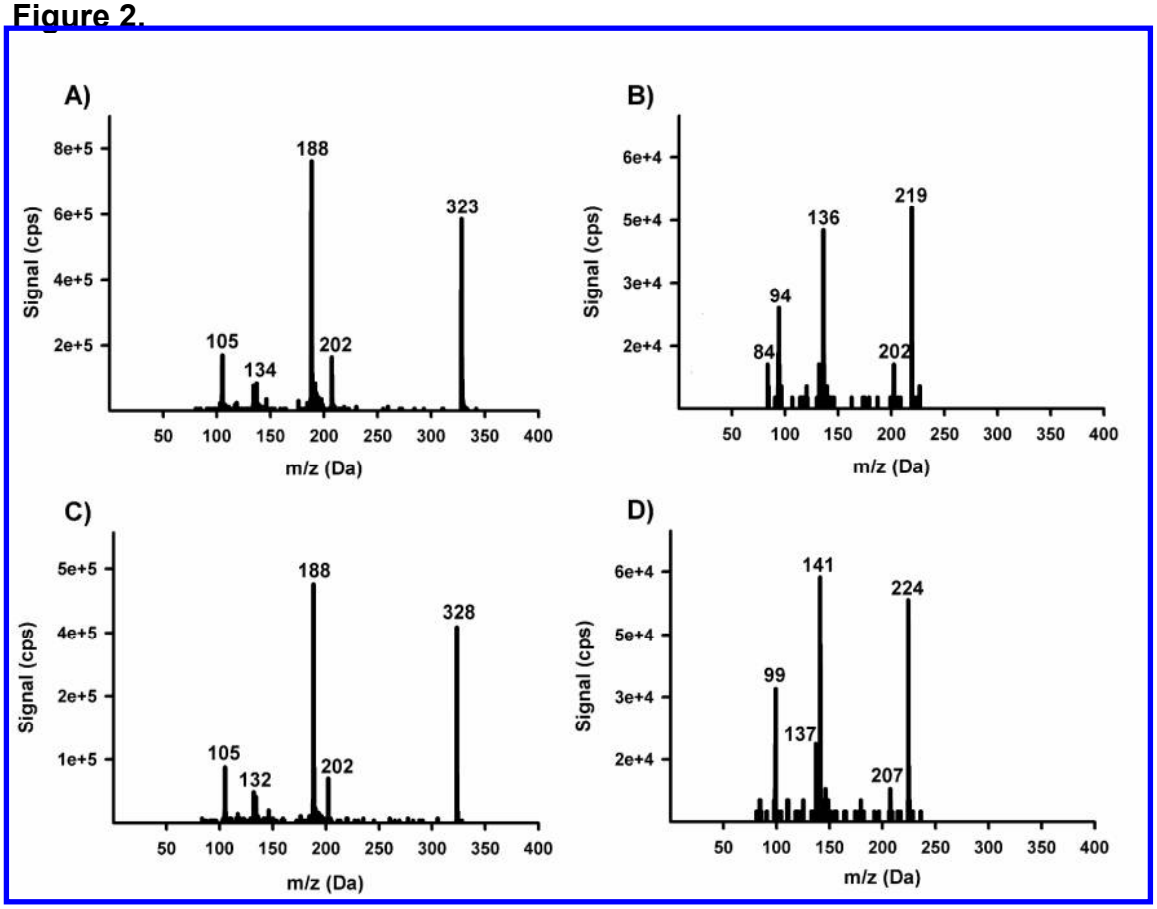

A)
Signal (cps)
8e+5
6e+5
4e+5
2e+5
188
323
105
134
202
50 100 150 200 250 300 350 400
m/z (Da)
B)
Signal (cps)
6e+4
5e+4
3e+4
2e+4
219
136
94
84
202
50 100 150 200 250 300 350 400
m/z (Da)
C)
Signal (cps)
5e+5
4e+5
2e+5
1e+5
188
328
105
132
202
50 100 150 200 250 300 350 400
m/z (Da)
D)
Signal (cps)
6e+4
5e+4
3e+4
2e+4
141
224
99
137
207
50 100 150 200 250 300 350 400
m/z (Da)

Figure 3.

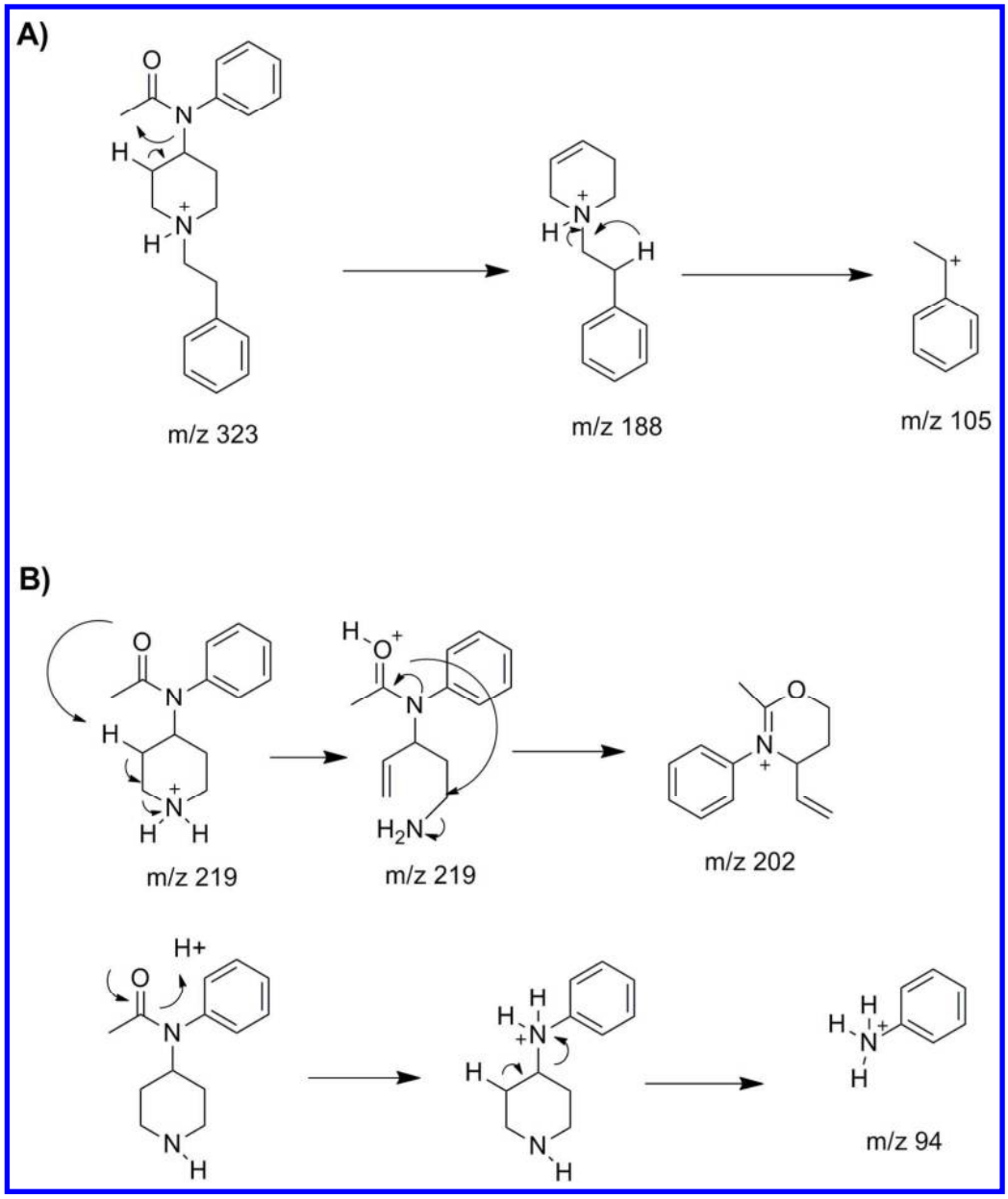


Figure 4.

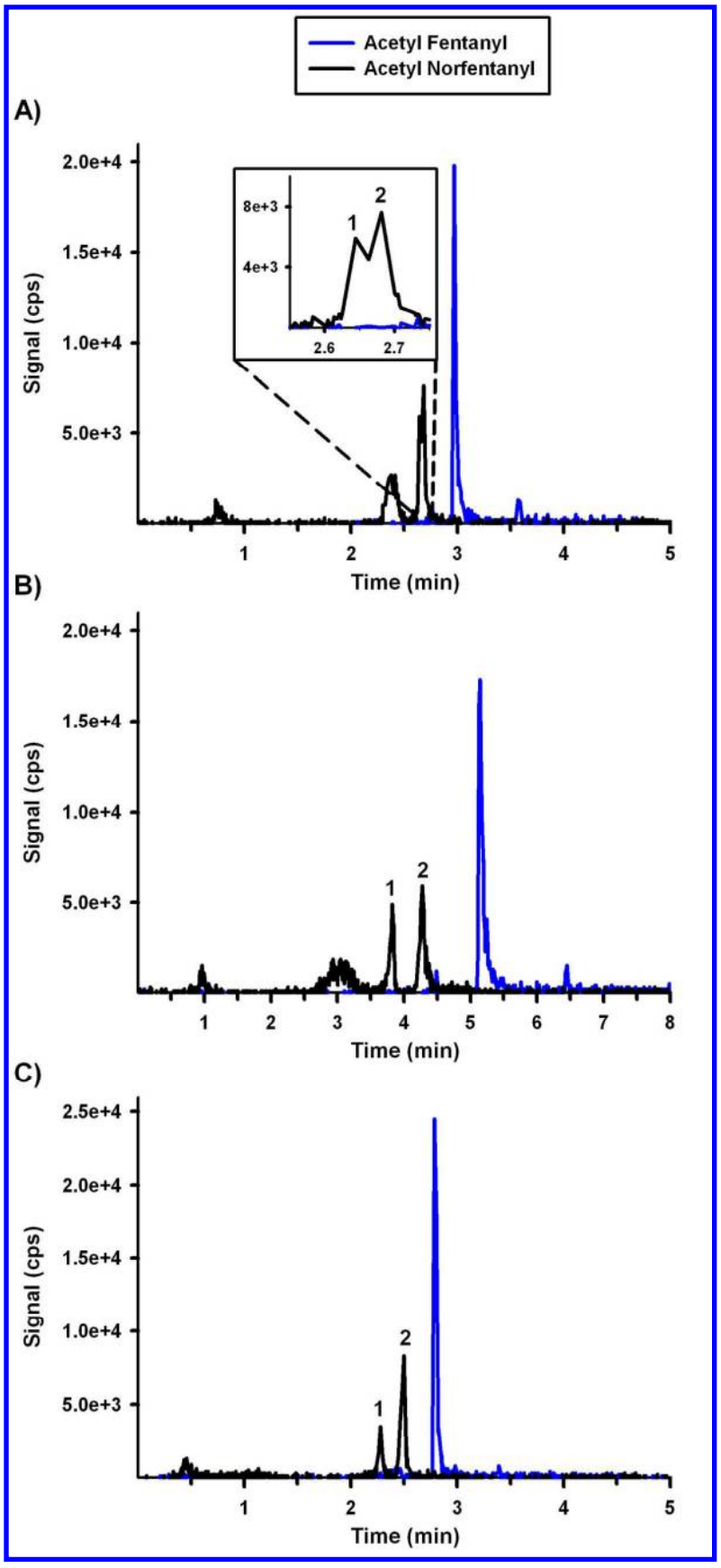

Acetyl Fentanyl
Acetyl Norfentanyl
A)
B)
C)
Signal (cps)
Time (min)
2.0e+4
1.5e+4
1.0e+4
5.0e+3
2.5e+4
8e+3
4e+3
2.6
2.7
1
2
3
4
5
6
7
8

Figure 5.

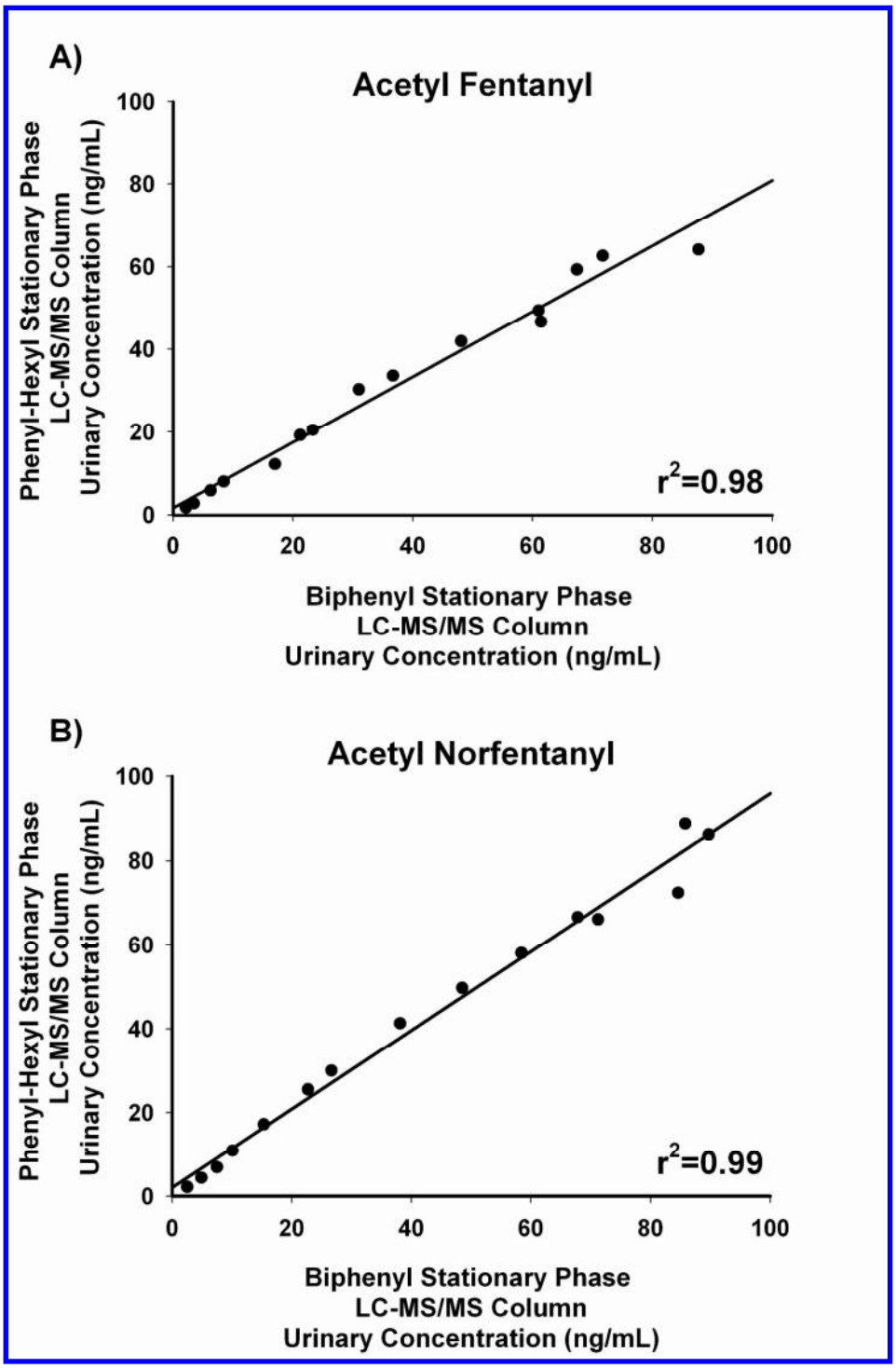

A)
Acetyl Fentanyl
Phenyl-Hexyl Stationary Phase
LC-MS/MS Column
Urinary Concentration (ng/mL)
r²=0.98
Biphenyl Stationary Phase
LC-MS/MS Column
Urinary Concentration (ng/mL)
B)
Acetyl Norfentanyl
Phenyl-Hexyl Stationary Phase
LC-MS/MS Column
Urinary Concentration (ng/mL)
r²=0.99
Biphenyl Stationary Phase
LC-MS/MS Column
Urinary Concentration (ng/mL)

Figure 6.

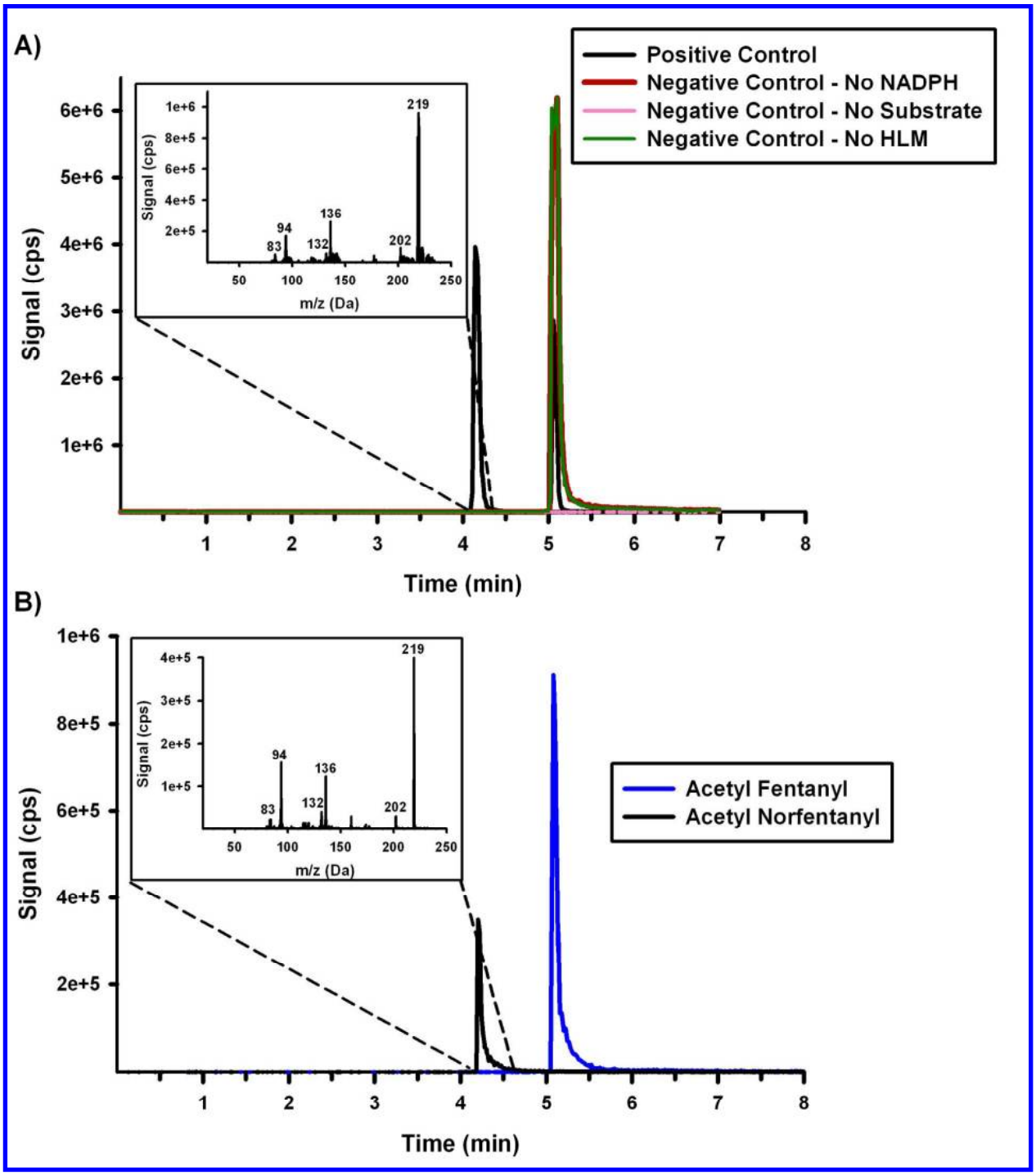

A)
Positive Control
Negative Control - No NADPH
Negative Control - No Substrate
Negative Control - No HLM
Signal (cps)
Time (min)
m/z (Da)
B)
Acetyl Fentanyl
Acetyl Norfentanyl
Signal (cps)
Time (min)
m/z (Da)

FIGURE LEGENDS

Figure 1. ^{1}H NMR confirmation data for (A) acetyl fentanyl, (B) acetyl norfentanyl, (C) acetyl fentanyl-d_5, and (D) acetyl norfentanyl-d_5 reference standards.

Figure 2. Representative mass spectra for (A) acetyl fentanyl, (B) acetyl norfentanyl, (C) acetyl fentanyl-d_5, and (D) acetyl norfentanyl-d_5 reference standards.

Figure 3. Proposed MS/MS fragmentation pathways for (A) acetyl fentanyl and (B) acetyl norfentanyl. The fragmentation products in the schematic correspond to the fragments observed in the mass spectra in Figure 2.

Figure 4. Representative chromatograms from analytical columns manufactured with phenyl-hexyl (A & B) and biphenyl (C) stationary phases. The different color tracings represent the specific reaction monitoring experiments used for each analyte. The notation “1” illustrates an isobaric interference that co-eluted with acetyl norfentanyl that is denoted as “2”. The chromatogram illustrated in panel A used the initial flow rate conditions of 800 µL/min, but as illustrated in panel B, a slower flow rate of 600 µl/min was required to resolve the isobaric interference. Data presented in panel C demonstrate that resolution of the isobaric interference is maintained at higher flow rates (800 µL/min) when using the biphenyl stationary phase.

Figure 5. A comparison of quantitative results obtained with liquid chromatography columns manufactured with phenyl-hexyl and biphenyl stationary phases. Acetyl

fentanyl (A) and acetyl norfentanyl (B) were simultaneously assayed as described in materials and methods. Fifteen human urine samples were spiked with concentrations spanning the calibration range of each analyte. Pearson correlation coefficients (r^2) are inset within each panel.

Figure 6. Representative chromatograms demonstrating that cytochrome P450 metabolizing enzymes in (A) human liver microsomes produce the predicted acetyl norfentanyl metabolite. The different color tracings represent combined extracted specific reaction monitoring (SRM) experiments optimized for the acetyl fentanyl substrate and acetyl norfentanyl metabolite. Specific conditions for each SRM are provided in the Experimental Section. Each reaction condition noted in the figure legend was run in triplicate and none of the negative controls generated measurable levels of the acetyl norfentanyl metabolite. Results obtained from urine collected from rats acutely exposed to acetyl fentanyl (3 mg/kg i.v.) produced similar results. The representative chromatogram illustrated in panel B were the results obtained from urine collected 3 hours post-administration. Quantitative results obtained for each time point are provided in Table 3.

TABLES

Table 1. Specific Reaction Monitoring (SRM) Experimental Parameters

Analyte	Q1 (*m*/*z*)	Q3 (*m*/*z*)	DP[a] (V)	EP[b] (V)	CE[c] (V)	CXP[d] (V)
Acetyl Fentanyl	323	188	101	10	35	8
	323	105	101	10	57	10
Acetyl Fentanyl-d5	328	188	56	10	33	4
	328	105	56	10	61	4
Acetyl Norfentanyl	219	84	71	10	25	12
	219	136	71	10	27	10
Acetyl Norfentanyl-d5	224	84	66	10	25	4
	224	141	66	10	29	10

[a]DP, declustering potential; [b]EP, entrance potential; [c]CE, collision energy; [d]CXP, collision cell exit potential

Table 2. Summary of accuracy, precision, and quantification limits in human urine*

	Quality Control High (75 ng/ml)				Quality Control Medium (7.5 ng/ml)				Quality Control Low (3.75 ng/ml)				
Analyte	**Conc. ± SD (ng/ml)**	**Inter-Day %CV[a]**	**Intra-Day %CV[a]**	**%RE[b]**	**Conc. ± SD (ng/ml)**	**Inter-Day %CV[a]**	**Intra-Day %CV[a]**	**% RE[b]**	**Conc. ± SD (ng/ml)**	**Inter-Day %CV[a]**	**Intra-Day %CV[a]**	**%RE[b]**	**LLQ**
Acetyl Fentanyl	77.7 ± 7.32	9.4	4.3	3.65	7.39 ± 0.59	7.9	5.0	1.43	3.71 ± 0.35	9.5	7.5	0.96	1.06
Acetyl Norfentanyl	76.8 ± 5.93	7.7	7.9	2.34	7.59 ± 0.44	5.8	3.8	1.19	3.84 ± 0.54	14.0	3.8	2.27	1.62

%CV[a], Coefficient of Variation; %RE[b], Absolute Relative Error; LLQ, Lower Limit of Quantification

*Data are based on 6 independent experiments conducted on multiple, nonconsecutive days

Table 3. Quantitative measurement of acetyl fentanyl and acetyl norfentanyl detected in rat urine collected after acute exposure to acetyl fentanyl (3 mg/kg i.v.)

	Rat Subject #1		Rat Subject #2	
Time	**Acetyl Fentanyl (ng/mL)**	**Acetyl Norfentanyl (ng/mL)**	**Acetyl Fentanyl (ng/mL)**	**Acetyl Norfentanyl (ng/mL)**
0 min	ND*	ND*	ND*	ND*
10 min	50.4	ND*	ND*	ND*
180 min	--	--	17300[#]	16500[#]
360 min	--	--	185[#]	3810[#]

*ND: Not detected

¨Rat 1 expired 15 min post-drug administration

[#]All samples were diluted within the range of the calibration curve

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