Rapid Analysis of Fentanyls and Other Novel Psychoactive Substances (NPS) in Substance Use Disorder Patient Urine using Paper Spray Mass Spectrometry

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Abstract

RATIONALE: Drug overdose deaths due to fentanyls and other novel psychoactive substances (NPS) are on the rise. The higher potencies of fentanyl analogs compared with morphine require new technologies to identify and quantitate NPS.
METHODS: Paper spray tandem mass spectrometry and high-resolution mass spectrometry were used to identify and measure fentanyl analogs as well as common drugs of abuse in urine samples from substance use disorder clinics. Ten-microliter urine samples were deposited directly on paper spray cartridges previously loaded with internal standards, dried, and analyzed with no other sample treatment. Quantitative results were obtained using tandem mass spectrometry (MS/MS). Individual drugs were identified using high resolution accurate mass spectrometry (HRAM), and confirmed by data dependent MS/MS.
RESULTS: Calibration curves in urine were linear over a range of 0.5 - 50 ng/mL with R^2 of 0.99 or better for eight representative fentanyl analogs. Cartridges preloaded with internal standards demonstrated satisfactory quantitative results compared with LC/MS. Direct identification and confirmation of fentanyl analogs and other common drugs of abuse in urine using high resolution accurate mass and MS/MS fragmentation were demonstrated at low picogram levels.
CONCLUSIONS: Paper spray mass spectrometry can reliably identify and quantitate fentanyl analogs and other drugs of abuse in urine. Using paper spray cartridges as collection devices reduces exposure and transportation risks associated with biological fluids. Cartridges pre-loaded with labeled internal standards can be effective for targeted screening of fentanyl analogs and other drugs of abuse.

KEY WORDS: Fentanyl Analogs, Paper Spray Mass Spectrometry, Novel Psychoactive Substances (NPS), Opioid Harm Reduction, High Resolution Accurate Mass Spectrometry (HRAM), Direct Quantitative Opioid Identification and Measurement

Introduction

Opioid related overdose deaths in the United States have risen more than four-fold from 2000 to 2016, with about half of this increase in the last five years.[1] About 60,000 fatal overdoses occurred in 2015 alone, with novel psychoactive substances (NPS) such as fentanyl analogs being identified as the leading cause.[2] NPS are a moving target as illustrated by structural modifications of fentanyl, a synthetic short-acting opioid with 50-100 times the potency of morphine that has long been utilized as an effective pain treatment.[3] The ease of synthesis of pharmaceutical fentanyl (PF) combined with facile modification of its basic structure by clandestine labs has resulted in a continuously growing number of related analogs.[4] For example, acetyl fentanyl (AcF) and acryl fentanyl are derived from a modified acylating reagent in the last step of synthesis, while sufentanil and carfentanil are synthesized by varying the starting piperidone moiety. These analogs are but a few examples of what has been termed Illicitly manufactured fentanyl (IMF) by the United States of America (USA) Drug Enforcement Agency (DEA) and Centres for Disease Control (CDC) agencies, and they are commonly sold as or mixed with heroin. A synthetic scheme for fentanyl with nomenclature for the different substituents that may be easily modified is illustrated in Figure 1 (summarized from[4]). This synthesis is readily available on the internet, and because of the ease of synthetic modification, a growing range of fentanyl analogs is expected in the future.[5]

A fentanyl immunoassay can detect analogs such as AcF in blood or urine. Because AcF and other analogs might not be detected by targeted, confirmatory MS testing, an unconfirmed screen positive may be erroneously dismissed.[6] The consequence of such apparent false positive screens was illustrated by a series of overdoses of AcF, an IMF found to be up to five times more potent than heroin in animal studies.[7] As a consequence of the dramatically increased distribution of IMF by criminal organizations since 2013[8], the DEA and CDC issued nationwide alerts in the USA in 2015, identifying IMF such as AcF as a threat to public health and safety. Nationally, the number of fentanyl submissions (*i.e.* samples obtained by law enforcement) and synthetic opioid deaths increased by 426% and 79% during 2013-2014, with fentanyl submissions being strongly correlated with increases in synthetic opioid deaths. A collaborative public health and law enforcement approach is being applied in the USA to counter this epidemic of fatalities: this includes implementing the CDC Guideline for Prescribing Opioids for Chronic Pain[9,10], improving access to and use of prescription drug monitoring programs, enhancing naloxone distribution and other harm reduction approaches, increasing opioid use disorder treatment capacity, improving linkage into treatment, and supporting law enforcement strategies to reduce the illicit opioid supply.[11,12] As of October 2017, there are no USA Substance Use and Mental Health Services Administration (SAMHSA) mandatory guidelines for federal workplace drug testing for fentanyls, although this is currently under review.[13]

Recognizing that heroin users have limited ability to identify fentanyls in their drugs, harm reduction strategies have included the use of drug testing dipsticks and methods such as Fourier Transform Infrared (FT-IR) spectroscopy. This approach shows promise in that users provided with information about the content of their drugs have been shown to respond with behaviors that reduce overdose risk.[14]

Unfortunately, the situation has been made more complex by the arrival of even more potent analogs such as carfentanil (approximately 100 times more potent than fentanyl[15]), which has been blamed for over 400 fatalities in the USA in 2017.[16] Such levels of potency mean that typical concentrations in street drugs are almost certainly below the detection limits of drug testing methods such as FT-IR or Surface Enhanced Resonance Raman Spectroscopy (SERS). These approaches have LODs of low percent quantities (*ca* >3% w/w) of fentanyl[14], and cannot reliably detect it at the levels typically found in street drugs. Moreover, such drugs are often found in conjunction with fentanyl itself, creating the risk of misleadingly positive results by a drug testing dipstick. A similar problem is presented by non-fentanyl opioid NPS such as U-47700 (potency about 7.5 times that of morphine)[15] which do not cross-react on an opiate or fentanyl dipstick, and may be below the detection limits of FT-IR.

A recent review of forensic drug testing methods and their suitability for harm reduction point-of-care services details a wide range of analytical methodologies. They predict that MS-based methods, the current gold standard in lab-based methods, hold great promise in the immediate future.[17] It is with these point-of-care harm reduction strategies in mind that some researchers are pursuing ambient ionization with MS methods, suitable for drug testing in real time, such as Direct Analysis in Real Time (DART)[18,19] or paper spray (PS)[20-22] ionization approaches. DART mass spectrometry (DART-MS), offers advantages for the sensitive, qualitative detection of drugs directly from the surfaces of a wide variety of substances, but it is challenging to use for accurate quantitative measurements. Paper spray mass spectrometry (PS-MS) can provide quite reasonable qualitative and quantitative information, but requires that the sample be deposited (as a fluid) on a paper sampling substrate, and the use of (labeled) internal standards, frequently spotted on the paper immediately prior to analysis.

Initially described in 2010, PS ionization allows for rapid analysis of samples without chromatography or prior purification, using a cellulose substrate shaped to have a fine tip.[23] When solvent and voltage are applied, ions are generated in a manner similar to those produced by an electrospray source.[24] A wide variety of sample types, such as powder or biological fluids, can be dissolved or extracted with common solvents and deposited directly on disposable PaperSpray® cartridges for high-throughput analysis without any sample cross contamination. The combination of PaperSpray® cartridge sample introduction, High Resolution Accurate Mass Spectrometry (HRAM) and tandem MS provides powerful and simple tools for reasonably robust identification of NPS, even in the absence of reference standards.[21,25]

The analysis of drugs of abuse (DOA) and other therapeutic drugs in biological fluids, (predominantly blood) using PS-MS has been documented in numerous publications.[21,22,26-32] The use of ambient ionization in conjunction with portable mass spectrometers and simplified operating systems has evolved significantly in recent years, to where point-of-care analysis by non-professional users is now a realistic goal. As an example, van Asten and co-workers reported a validated method for eight amphetamines in whole blood using PS-MS, as well as plans to transfer the approach to a portable mass spectrometer for onsite monitoring.[33]

Despite these advances, to date, few manuscripts have reported the analysis of fentanyl or its analogs using PS-MS.[20,21] In this paper, we present methods for the identification and measurement of fentanyl and its analogs, as well as other common drugs of abuse in anonymous urine samples from substance use disorder clinics, using PaperSpray® cartridge based ionization with both tandem mass spectrometry (MS/MS), and high resolution accurate mass spectrometry (HRAM) coupled with data dependent MS/MS fragmentation. In addition, to utilizing this simplified biological fluid sample collection strategy, we evaluated the use of these cartridges pre-loaded with labeled internal standards, sending them to a remote location for urine sampling.

Experimental

Instrumentation

A Velox® 360 PaperSpray® system (Prosolia Inc., Indianapolis, IN, USA) was coupled to either a triple quadrupole mass spectrometer (Model TSQ, Thermo Fisher Scientific, San Jose, CA, USA) or a high-resolution mass spectrometer (Exactive Focus Orbitrap, Thermo Fisher) for PS-MS analysis. HPLC/MS analysis employed a 2.1 × 50 mm Kinetex Biphenyl column (Phenomenex, Torrance, CA, USA) with a binary pump liquid chromatograph (1200 Series, Agilent Technologies, Santa Clara, CA, USA) interfaced to a triple quadrupole mass spectrometer (Model 6410, Agilent Technologies).

Reagents and Samples

Acetylfentanyl, acrylfentanyl, fentanyl, carfentanil and sufentanil, principal metabolites norfentanyl, norcarfentanil and desmethyl U-47700 (a metabolite of the opioid U-47700) and internal standards acetylfentanyl-$^{13}C_6$, fentanyl-d_5, norfentanyl-d_5, carfentanil-d_5, sufentanil-d_5 and desmethyl U-47700-d_3 were purchased from Cerilliant (Round Rock, TX, USA). Although these commercially available standards are registered test kits at trace levels, with legal exemptions for their use and shipment in both Canada and/or the United States, suitable personal protection should be used when preparing standards to avoid exposure. Diluted stock solutions were volumetrically prepared (10000 ng/mL) in methanol using calibrated pipettes. Standards were prepared by serial dilution of the stock in unfiltered human urine over the concentration range of 0.5 to 50 ng/mL. Labeled internal standards were prepared as a cocktail at concentrations of 20 or 50 ng/mL in methanol using calibrated pipettes. Mannitol and acetic acid (ACS Grade) and acetonitrile, methanol and water (HPLC Grade) were purchased from VWR (Radnor, PA, USA). Anonymous urine samples were obtained from LifeLabs Medical Laboratories (Burnaby, BC, Canada) where they had been sent from substance use disorder clinics for fentanyl testing.

PS-MS Measurements

PaperSpray® cartridges for the Velox® 360 system were commercially obtained (Prosolia Inc.) and used without further treatment or cleaning. Prior to shipment, 10 µL of a 10% (w/v) aqueous solution of mannitol was added to the cartridges and dried prior to the addition of 10 µL of an internal standard cocktail, allowing the cartridge to dry at room temperature (Indianapolis, IN, USA). Following

air shipment of the cartridges to the remote testing laboratory (Vancouver Island, BC, Canada), 10 µL of anonymous urine samples (previously analyzed by HPLC/MS for NPS, procedures used published elsewhere[34]) were deposited on the preloaded cartridges, which were then returned by air shipment for PS-MS measurements. For clarity, the anonymous urine samples were originally submitted to LifeLabs for standard drug testing, including fentanyl and its analogs. The presented study was part of ongoing research and development, and therefore exempt from ethics approval considerations. A 90/9.9/0.1 (v/v) mixture of acetonitrile / water/ acetic acid was used as both the PS-MS extraction and elution solvent, with an applied voltage of 4 (Thermo Model TSQ) or 5 (Thermo Exactive) kV used to generate ions for analysis. In either case, ions were generated by applying high voltage for 48 seconds, integrating the total signal obtained (*i.e.* area under curve, AUC) for the appropriate MS/MS transition of each analyte or internal standard. The analytical duty cycle for a typical measurement is <1 min/sample. Calibration curves over a range of 0.5 - 50 ng/mL were generated for each compound via MS/MS, using area ratios with the corresponding internal standard. A summary of MRM conditions for the fentanyl analogs used in PS-MS analysis on the triple quadrupole mass spectrometer is available in the supplementary information (Table S1).

Analytical standards of the 7 fentanyl analogs examined (0.2 ng each) were deposited on paper spray cartridges, and analyzed by data-dependent scanning using the high-resolution mass spectrometer. Rapid screening and identification of fentanyl and other DOA were accomplished using accurate mass measurements and confirmed by data dependent MS/MS scanning from an inclusion table implemented with the high-resolution MS system. First, the accurate mass full scan data was obtained. For any mass present in the full scan that matched an analyte mass in the inclusion table, a confirmatory MS/MS experiment was also conducted. The complete inclusion table for all target NPS analytes examined is given in the supplementary information (Table S2).

Results and Discussion

Mannitol Ionization Enhancement

When investigating possible matrix affects, mannitol, a common additive used to dilute street drugs, was found to enhance the signal areas and calibration sensitivity (31% calibration slope increase) for acetylfentanyl, as shown in Figure 2. Similar enhancements were also observed for fentanyl and other analogs. Consequently, 10 µL of a 1.0% w/v solution of mannitol in water was added to the cartridges for all subsequent experiments to enhance ionization. At the time of the study, we did not have the necessary legal exemptions for research utilizing authentic street drug samples. Since the amount of mannitol in street drugs is unknown, additional experiments to study the effect of mannitol concentration could not be performed, but this aspect will be examined in future research.

Quantitative MS/MS Method Development

Linearity data over a concentration range of 0.5 to 50 ng/mL in urine was obtained using PS-MS for each of the analytes, measuring each standard in triplicate. An example of a typical signal trace obtained for fentanyl in urine using PS-MS is given in Figure S1 (supplementary information). A summary of the method

development data for all analytes is included in Table 1 (example calibration curves for selected NPS are given in Figure S2, supplementary information). The % bias was determined based upon the difference between the calculated and known concentrations of the analytical standards. The reported % bias at 2.5 ng/mL and 50 ng/mL provides accuracy estimates for the method based upon known analyte concentrations in urine. Least squares regression linear fits and R^2 values were determined from the calibration curves over a concentration range of 0.5 to 50 ng/mL. The reported RSD was calculated from 8 replicates of a 10 ng/mL spiked urine sample. Table 2 gives a summary of the average signal to noise ratios obtained for measurements of 0.5 ng/mL standards in urine (n=5 for each). This was the lowest measured concentration in the study. Based upon these results, conservatively estimated detection limits for the target analytes using this PS-MS method are *ca* 0.5 ng/mL in urine.

Using these results, fentanyl and norfentanyl concentrations were determined for 35 anonymous urine samples from substance use disorder clinics, using both PS-MS (0.5 - 50 ng/mL calibrator range) and HPLC/MS (1 to 40 ng/mL calibrator range), comparing the results graphically in a scatter plot (Figure 3). Although the levels of fentanyl and norfentanyl in many of the urine samples were well above the highest calibrators, the agreement between the two methods (indicated by the strong correlation coefficients and near unity slopes) is sufficient for clinical harm reduction purposes. The pre-spiking of labeled internal standards and dried urine samples, and their shipment time (*ca* 10 days) did not result in significant analytical bias, suggesting that the preparation and distribution of assay specific PS-MS cartridges is feasible. Samples with norfentanyl >1000 ng/mL were not included in these comparisons. Analytical calibration ranges are biased towards lower concentrations, as these are characteristic of remote ingestion (*i.e.* use in the past), which is the scenario most likely to be questioned by both patient and healthcare practitioner.

HRAM and Data Dependent MS/MS for NPS Identification

To further illustrate the use of PS-MS for rapid NPS detection and identification, selected anonymous urine samples were also analyzed using high resolution accurate mass spectrometry (HRAM), based upon the hypothesis that those with higher concentrations of fentanyl were more likely to also contain other drugs of abuse (DOA) or fentanyl analogs. This assumption was based upon clinical laboratory observations that fentanyl analogs are only rarely detected in urine in the absence of fentanyl (unpublished results). Initially, neat samples of the 7 fentanyl analogs were deposited at low concentration (10 µL of a 0.2 ng/mL standard cocktail in methanol, 2 pg of analyte) on PaperSpray® cartridges, and examined by data dependent scanning on the high-resolution mass spectrometer, collecting both HRAM and MS/MS spectra. Data dependent MS/MS and HRAM spectra for fentanyl and carfentanil are presented in Figure 4, illustrating their detection at ppb levels using PS-MS. The MS/MS results are consistent with previously published fragmentation patterns for both compounds.[35] Consequently, PS-MS can potentially identify trace levels of highly potent opioid analogs such as carfentanil, which are likely to escape detection in harm reduction by dipsticks and FT-IR methods.

Based upon satisfactory results obtained for low for ppb level standards (Figure 4), selected urine samples chosen from the pool were examined by the same approach. A wide variety of common DOA as well as fentanyl and norfentanyl were

conclusively identified based on HRAM as well as MS/MS fragmentation data. As an example, the HRAM mass spectrum for clinical urine sample AERL5 is given in Figure 5, demonstrating the presence of methamphetamine, fentanyl, norfentanyl, methadone, and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP, the major methadone metabolite). Identity assignment based on accurate mass was confirmed in each case by data dependent MS/MS analysis (Figure S3, supplementary information).

The combination of HRAM and MS/MS was used to confirm the presence of other drugs of abuse in the clinical urine samples. A summary of the results obtained for selected samples is presented in Table 3. Methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA) and cocaine were the most common DOA identified in these urine samples, in addition to fentanyl and norfentanyl. Cyclopropylfentanyl, a 'new' IMF[34], was detected in several samples, with only one sample testing positive for norcarfentanil. Based upon comparison with signals observed for neat analytical standards, acetylfentanyl, acrylfentanyl, carfentanil, desmethyl U-47700 and sufentanil were not detected in any of the urine samples analyzed.

Conclusions

The presented work illustrates that PS-MS can be used to screen, identify and provide quantitative results fentanyl and other common DOA in urine. HRAM and MS/MS spectra derived from PS ionization are effective tools for identifying drugs when reference standards are not readily available. Preloaded PaperSpray® cartridges with labeled internal standards can be utilized in targeted analysis for fentanyl analogues and other DOA, with the viable option of conducting such testing at the point-of-care in a clinic or mobilized in-field setting. The use of PaperSpray® cartridges as sample collection devices reduces the hazards associated with and simplifies the transportation of biological fluid samples. Immediate future research includes the development and implementation of point-of-care style PS-MS systems for use in forensic, first responder and clinical harm reduction strategies (*i.e.* drug testing in supervised safe use sites).

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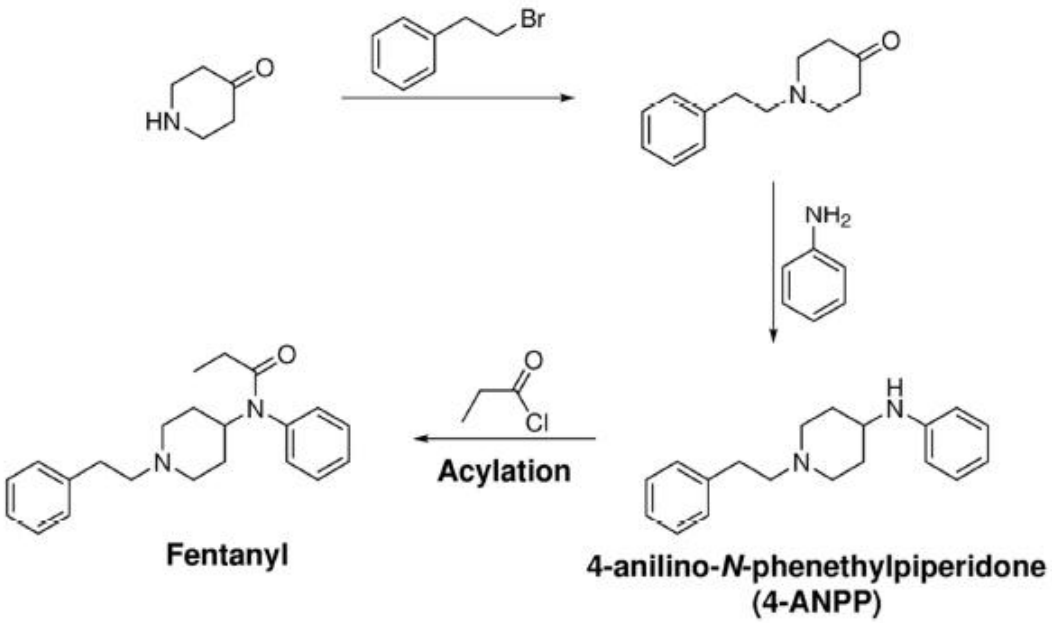

Figure 1. Reaction scheme for the synthesis of fentanyl.

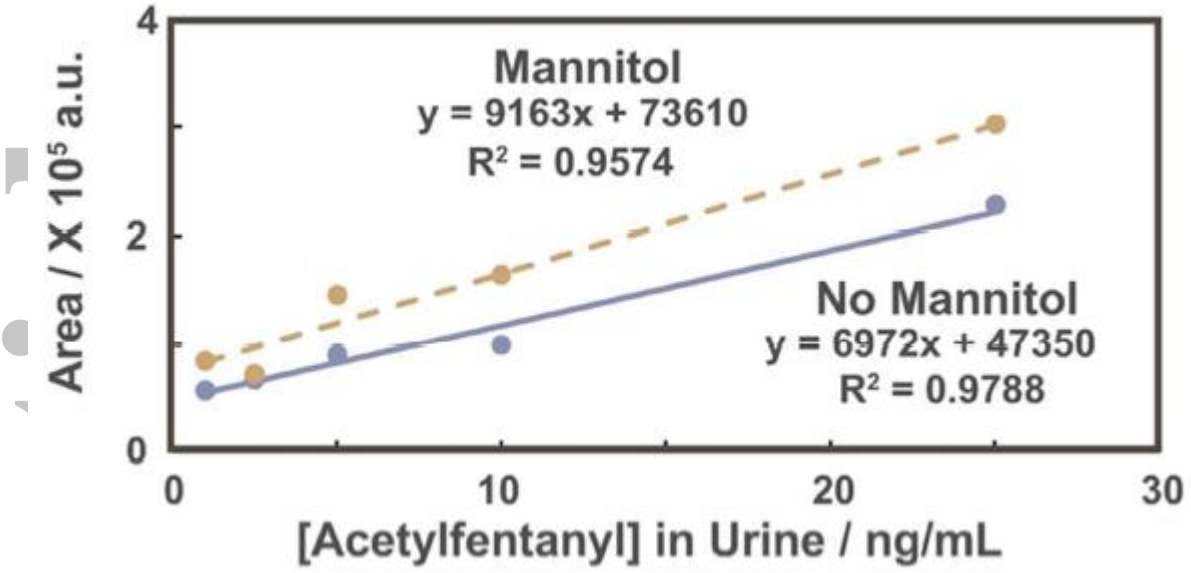

Figure 2. PS-MS signal enhancement for observed for acetylfentanyl induced by the addition of mannitol.

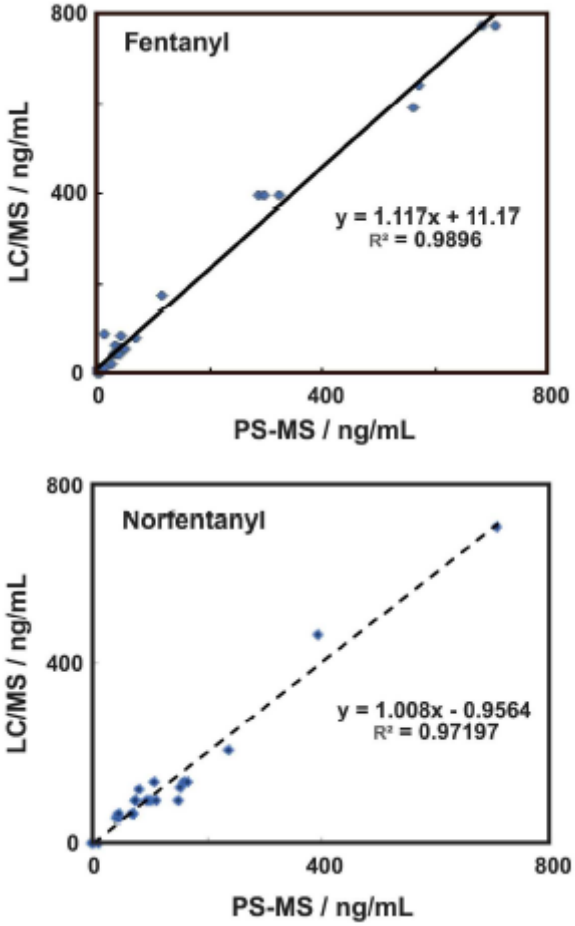

Figure 3. Comparison of results obtained for fentanyl and norfentanyl measurement in anonymous urine samples by PS-MS and HPLC/MS obtained from a substance use disorder clinic.

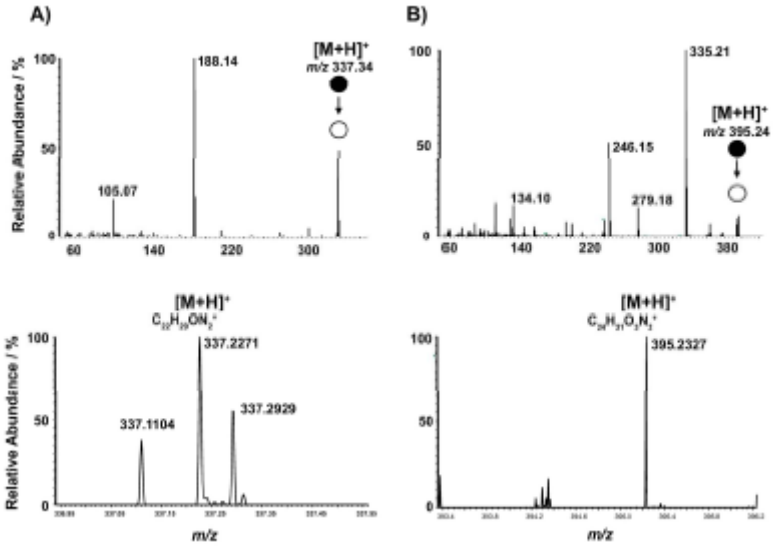

Figure 4. HRAM (bottom panels) and data dependent MS/MS (top panels) PS-MS spectra obtained for the direct measurement of a standard cocktail containing 2 pg of (A) fentanyl and (B) carfentanil.

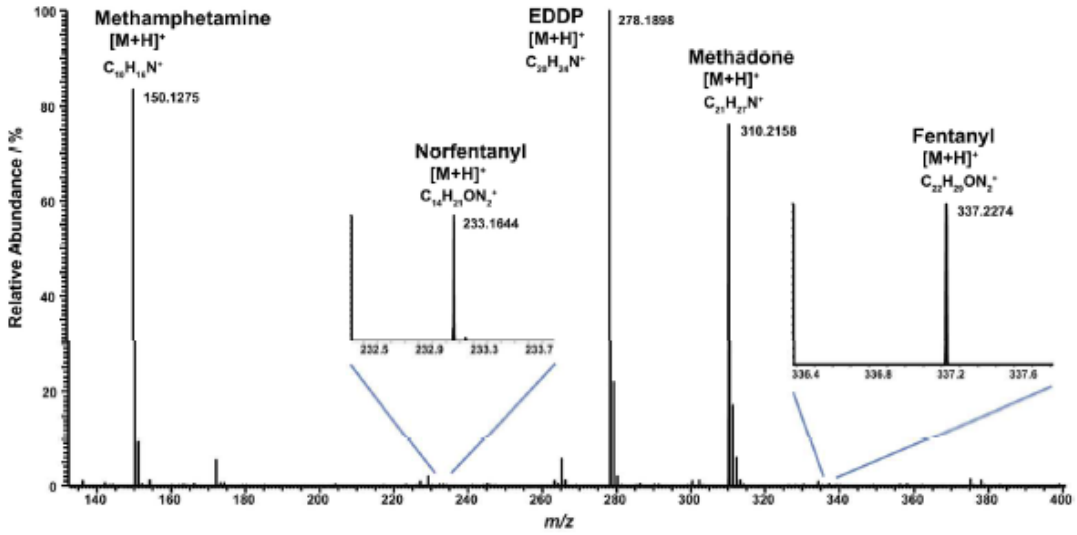

Figure 5. HRAM spectrum for a clinical urine sample, demonstrating the presence of methamphetamine, fentanyl, norfentanyl, methadone, and EDDP. Confirmatory data dependent MS/MS spectra for all HRAM assignments are given in the supplementary information (Figure S3).

Table 1. Summary of Method Development for the Quantitative Paper Spray Analysis of Fentanyl Analogs spiked in Urine.

	% Bias for 2.5 ng/mL	% Bias for 50 ng/mL	Least Squares Regression Fit	R^2	RSD
Acetylfentanyl	11	2	Y=0.0171X+0.0403	0.996	7.1
Acrylfentanyl	8	2	Y=0.0202X+0.0135	0.997	6.4
Carfentanil	5	2	Y=0.078X+0.1182	0.996	10.1
Norcarfentanil	24	3	Y=0.0167X-0.0013	0.993	14.8
Fentanyl	16	4	Y=0.0105X+0.0138	0.990	5.4
Norfentanyl	16	3	Y=0.0106X-0.0088	0.991	11.3
Sufentanil	26	3	Y=0.0756X-0.0241	0.994	6.9
Desmethyl U-47700	48	3	Y=0.0095X+0.0683	0.989	6.3

Table 2. Summary of average S/N values for 0.5 ng/mL standards spiked in urine (n=5 for each).

Analyte	S/N for 0.5 ng/mL
Acetylfentanyl	56
Acrylfentanyl	53
Carfentanil	84
Norcarfentanil	39
Fentanyl	37
Norfentanyl	20
Sufentanil	67
Desmethyl U-47700	56

Table 3. Summary of HRAM and data dependent MS/MS identification of drugs of abuse in selected urine samples obtained from a substance use disorder clinic. For clarity, abbreviations used include: Fentanyl (Fen), Norfentanyl (NFen), Norcarfentanil (NCar), Cyclopropylfentanyl (CpFen), Methamphetamine (Meth), 3,4-Methylenedioxymethamphetamine (MDMA), 3,4-Methylenedioxy-*N*-ethylamphetamine (MDEA), and Oxymorphone (OxyM).

Urine Sample	Fen	NFen	NCar	CpFen	Meth	MDMA	MDEA	Cocaine	OxyM
AERL 2	x	x	x		x	x	x		
AERL 41	x	x			x	x			
AERL 42	x	x			x	x	x		
AERL 4	x	x			x				
AERL 5	x	x		x	x	x	x		
AERL 37	x	x		x	x				
AERL 16	x	x			x	x	x		
AERL 21	x	x			x	x			
AERL 24									x
AERL 20		x			x	x		x	
AERL 31	x	x		x	x	x	x	x	
AERL 32	x	x		x	x			x	
AERL 33	x	x		x	x	x	x	x	

x denotes confirmed identification in a urine sample by HRAM and MS/MS.