A Novel Oral Fluid Assay (LC-QTOF-MS) for the Detection of Fentanyl and Clandestine Opioids in Oral Fluid After Reported Heroin Overdose

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Abstract

Introduction The adulteration of heroin with non-pharmaceutical fentanyl and other high-potency opioids is one of the factors contributing to striking increases in overdose deaths. To fully understand the magnitude of this problem, accurate detection methods for fentanyl and other novel opioid adulterant exposures are urgently required. The objective of this work was to compare the detection of fentanyl in oral fluid and urine specimens using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in a population of heroin users presenting to the Emergency Department after overdose.

Methods This was a prospective observational study of adult Emergency Department patients who presented after a reported heroin overdose requiring naloxone administration. Participants provided paired oral fluid and urine specimens, which were prepared, extracted, and analyzed using a dual LC-QTOF-MS workflow for the identification of traditional

and emerging drugs of abuse. Analytical instrumentation included SCIEX TripleTOF® 5600+ and Waters Xevo® G2-S QTOF systems.

Results Thirty participants ($N = 30$) were enrolled during the study period. Twenty-nine participants had fentanyl detected in their urine, while 27 had fentanyl identified in their oral fluid (overall agreement 93.3%, positive percent agreement 93.1%). Cohen's Kappa (k) was calculated and demonstrated moderately, significant agreement ($k = 0.47$; p value 0.002) in fentanyl detection between oral fluid and urine using this LC-QTOF-MS methodology. Additional novel opioids and metabolites, including norfentanyl, acetylfentanyl, and U-47700, were detected during this study.

Conclusion In this study of individuals presenting to the ED after reported heroin overdose, a strikingly high proportion had a detectable fentanyl exposure. Using LC-QTOF-MS, the agreement between paired oral fluid and urine testing for fentanyl detection indicates a role for oral fluid testing in surveillance for nonpharmaceutical fentanyl. Additionally, the use of LC-QTOF-MS allowed for the detection of other clandestine opioids (acetylfentanyl and U-47700) in oral fluid.

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Introduction

The addition of clandestine opioids (Table 1) to heroin produces an extremely potent drug mixture contributing to the striking increase in overdose deaths in the USA [1–3]. Commonly available drug tests, however, do not detect these clandestine opioids [4–6]. As a result, only limited data are available to confirm when (and which) clandestine opioids

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Table 1 Library of clandestine opioids available at the time of testing*Fentanyl analogs*

Acetylfentanyl
 Alpha-methylfentanyl
 3-Methylfentanyl
 Butyrylfentanyl
p-Fluorofentanyl
 Beta-hydroxythiofentanyl
 Valeryl fentanyl
 Furanyl fentanyl
 Carfentanil
 Norfentanyl (metabolite)

Non-fentanyl-related analogues

U-47700
 U-50488
 AH-7921
 MT-45

The addition of clandestine opioids to heroin produces an extremely potent drug mixture contributing to the striking increase in overdose deaths in the USA

contribute to overdoses. Current data on these lethal adulterants arise primarily from law enforcement opioid seizures and post-mortem testing following overdose deaths [7–12]. Law enforcement seizures are an important data stream, but are not linked to individual patients, patterns of use, or outcomes. Moreover, traditional post-mortem data can lag for months after a new clandestine opioid enters the heroin market [7]. This delay impedes clinical practice, public health response, addiction services delivery, and law enforcement efforts to save lives.

Nimble and reproducible surveillance methods are urgently required to keep pace with the emergence of clandestine opioids, inform clinical practice, and accelerate public health responses [13]. Due to privacy issues and increased risk of urine adulteration or substitution, an alternative matrix for which collection can be directly observed (e.g., oral fluid) has advantages in environments ranging from drug treatment clinics to roadside testing. Favorably, the window of detection for drugs in oral fluid tends to be more indicative of recent use, in contrast to urine in which residual drug may be detectable for several days after last exposure. In monitoring for esoteric and novel drug use, testing methods must be carefully selected, as traditional drug testing (such as immunoassays) and limited mass spectral libraries restrict the ability to detect fentanyl, its analogs, and other emerging drugs (e.g., due to lack of cross reactivity on immunoassay platforms) [14].

An innovation in clinical and forensic drug testing, liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) has the potential to increase sensitivity and scope of analytical testing for traditional and clandestine

opioids. In this assessment, an evaluation was conducted to determine if oral fluid testing would be comparable to urine testing for the detection of fentanyl use as determined by LC-QTOF-MS analysis.

Methods

A prospective, observational study was performed on adults who presented to the Emergency Department (ED) after heroin overdose requiring naloxone for reversal of respiratory depression. After informing participants about the study, an investigator obtained verbal consent, then asked participants to provide oral fluid and urine specimens for drug testing. No identifiable information was collected, and all study data (specimens and survey responses) were marked only with an anonymized study identification number. The study protocol was approved by an Institutional Review Board (IRB) and the Department of Emergency Medicine.

Study Setting and Participants

This study was conducted at an urban, medical school-affiliated, adult ED in New England (USA). Both the adult and pediatric EDs are level-one trauma centers, providing medical care to greater than 80,000 patients per year. Throughout the study period, three physicians and a research assistant enrolled participants during pre-selected dates and times. Our study population included consenting, English-speaking adults (≥ 18 years of age) who presented to the ED following a reported heroin overdose in which naloxone was administered. Patients who were intoxicated, critically ill, unable to make urine, or currently under police custody were excluded from this study.

During enrollment periods, study investigators queried the ED tracking system to identify eligible participants. Once pre-screened, individuals were approached by a member of the study team to further verify eligibility. No incentives were offered to participants, and study investigators were not directly involved in their clinical care.

Specimen Collection and Handling

Participants provided an unsupervised urine specimen, with a minimum of 10 mL of urine required for analysis. Once the urine specimen was obtained, investigators collected an oral fluid specimen from participants using a Quantisal® oral fluid collection device (Immunalysis Corporation; Pomona, CA, USA). The Quantisal® device consists of a cotton swab with an absorptive pad and volume adequacy indicator, 3 mL of a storage buffer, and a transport tube. The volume indicator changes color during sample collection to indicate that 1 mL ($\pm 10\%$) of oral fluid had been obtained. The swab was

maintained in the mouth of the participant until indicator color change occurred, or at least 10 min had elapsed. Once color change was achieved, or sufficient time had elapsed, the device was placed in the accompanying transport tube containing the buffer. All collection devices were labeled with a unique study identification number that linked the urine specimen to the oral fluid specimen from the individual participant. Collected specimens were placed in two impermeable bags and stored at 25 °C until shipping. Samples were shipped via overnight mail to the Center for Forensic Science Research and Education (CFSRE, Willow Grove, PA, USA) weekly, where they were stored at 4 °C and analyzed within 1 week of collection.

Analytical Testing

All testing was conducted at the CFSRE. Two aliquots of the same specimen were prepared for analysis using a single-step (pH 10.4) liquid-liquid extraction. Instrumental analysis was performed by two separate LC-QTOF-MS analytical methods, with distinctive LC separation and mass acquisition to indicate screening and confirmations of the analytes in the biological specimens. This protocol was previously validated for confirmatory qualitative analysis in blood, urine, and oral fluid specimens for clinical and forensic applications [15]. Verification was performed using opioid standard reference material (including fentanyl, norfentanyl, acetylfentanyl, carfentanil, U-47700, etc.) in extracted and unextracted forms, from applicable biological specimens as previously stated. Studies were conducted by serial dilution of standard reference material for determination of threshold detection limits (e.g., limits of detection, LOD): fentanyl 1 ng/mL, norfentanyl 2 ng/mL, acetylfentanyl 1 ng/mL, carfentanil 1 ng/mL, and U-47700 1 ng/mL.

Data processing was performed using appropriate manufacturer software for the identification of toxicologically relevant compounds. Processing was performed against extensive in-house databases that include exact mass, retention time, and exact mass fragments or library spectra. Positive drug identifications were made based on pre-established processing criteria, consistent with industry standards, including mass error (< 10 ppm), retention time (< 0.35 min difference), and fragments identified or library score (> 70). Confirmation was determined based on LC-QTOF-MS platform agreement.

Data Analysis

All statistical data analyses were performed using SAS® software (version 9.4), and two-sided *p* values below 0.05 were considered significant. Cohen's Kappa statistic (*k*) and *p* value were calculated to determine whether the level of agreement between oral fluid and urine testing results for fentanyl detection was a chance phenomenon.

Results

Participants were enrolled in this study between 24 August 2016 and 11 December 2016. During this timeframe, 21,061 patients were treated in the ED. During our pre-selected enrollment periods, 55 patients received a diagnosis of heroin overdose and were considered eligible. During the study period, 32 eligible individuals were approached for consent by a member of the research team. Thirty consented to study participation (93.8%); two individuals declined to participate. The demographic characteristics of participants can be found in Table 2.

All the enrolled participants reported heroin use, with injection use reported by 22 (73.3%), intranasal use reported by 7 (23.3%), and one individual reporting use by both routes (3.3%). All our participants reported an intent to purchase/use heroin; all denied seeking fentanyl. No participants reported prescription or non-prescription fentanyl use.

Of the 30 participants enrolled in this study, fentanyl was detected in the urine of 29 participants and the oral fluid of 27 participants (see Table 3). Paired analytical testing results were in agreement for 28 (93.3%) of the 30 paired specimens; fentanyl was detected in both matrices for 27 participants (positive percent agreement 93.1%), and undetected in both matrices for one participant. A Cohen's Kappa statistic of 0.47 and a *p* value of 0.002 demonstrated moderate, but significant, agreement between the two matrices for the detection of fentanyl using the above described analytical method.

Norfentanyl was detected in urine specimens collected from 28 participants; however, only two participants had norfentanyl detected in their oral fluid. Acetylfentanyl was detected in urine specimens of nine subjects, with consistency in four-paired oral fluid specimens. One individual had acetylfentanyl detected in their oral fluid only (see Table 4), and two participants had U-47700 detected in paired oral fluid and urine specimens.

Discussion

This study examined the potential for oral fluid to be used as an alternative matrix to urine for the detection of fentanyl after reported opioid overdose. In our study population, fentanyl was detected in the urine of 96.7% of the patients presenting after overdose and a high level of overall agreement (93.3%) was demonstrated between oral fluid and urine testing results for fentanyl by LC-QTOF-MS analysis. Additionally, using this methodology, novel clandestine opioids, acetylfentanyl and U-47700, were detected in oral fluid and urine specimens.

The detection of fentanyl in an individual's urine after overdose does not pinpoint fentanyl as the culprit, since any fentanyl exposure over the preceding 72 h may lead to a positive

Table 2 Demographic characteristics of participants

	<i>N</i> = 30
Age (years)	
Median (IQR)	20 (17–23)
Sex (%)	
Male	45.4
Female	54.6
Ethnicity/Race (%)	
White, non-Hispanic	86.7
Black, non-Hispanic	0.0
Hispanic	13.3
Other	0.0

All the enrolled participants reported heroin use, with injection use reported by 22 (73.3%), intranasal use reported by 7 (23.3%), and one individual reporting use by both routes (3.3%)

finding [16]. Additionally, the detection of fentanyl may be due to medically indicated use, the diversion of pharmaceutical fentanyl, or the exposure to non-pharmaceutical fentanyl; none of the patients in this study reported medical use or diversion of pharmaceutical fentanyl. The shorter window of detection for many drugs in oral fluid when compared to urine increases the utility of oral fluid to show more recent drug use; however, the specific window of detection for fentanyl and clandestine opioids in oral fluid is currently unknown [17].

One of the analytical challenges posed by oral fluid is the low volume of specimen available for testing by the laboratory (typically < 4 mL total) and the low-level concentrations of drugs in comparison to urine [16, 18]. These lower concentrations require adequately sensitive analytical platforms, such as LC-QTOF-MS, to ensure comprehensive and accurate detection. Testing strategies including HPLC and LC-MS/MS have been used successfully to detect fentanyl in oral fluid previously [19, 20]. However, broad-based screening for clandestine opioids by LC-QTOF-MS offers advantages over traditional

analytical methods. First, the identification of new substances by LC-QTOF-MS is more facile than LC-MS/MS due to non-targeted LC-QTOF-MS acquisition modes (e.g., MS/MS^{ALL} and MS^c). This allows for the detection of all compounds within a given mass range, regardless of incorporation into the existing library database, and the analysis of specimens via a central analytical method, alleviating the lengthy method development stages associated with LC-MS/MS analyses. Second, the digital and comprehensive nature of the stored data files and associated mass spectra allows for retrospective querying of a cohort of patients without re-analysis of specimens, an attribute extremely beneficial to clinical and forensic toxicology laboratories.

The presented findings are consistent with the known pharmacokinetics and pharmacodynamics of fentanyl. With a pK_a of 8.4, fentanyl remains ionized at physiologic pH and diffuses across plasma concentration gradients into oral fluid. Like other opioids, fentanyl (a weak base) may be concentrated by ion trapping in the mildly acidic oral fluid matrix [21]. Moreover, fentanyl acts as a substrate for *p*-glycoprotein with a postulated active transport mechanism for fentanyl and other opioids into saliva [19, 22]. Finally, fentanyl is a lipophilic drug which adheres well to the cotton-based applicators of most commercial oral fluid swabs [23].

For norfentanyl, a high level of discordance was found. Norfentanyl is the primary *in vivo* metabolite of fentanyl. In other work, norfentanyl has not been routinely detected in post-mortem blood specimens, despite the presence of high concentrations of fentanyl in the matrix [24]. Our relative inability to detect norfentanyl in oral fluid when compared to urine is similar to prior findings based on LC-MS/MS analysis [20]. It is suspected that norfentanyl detection is limited by the low concentrations present in oral fluid and the dilution inherent in Quantisal® collection. Also, the degree of adsorption of norfentanyl to the Quantisal® collector is unknown.

In this study, the threshold limits of detection (LOD) for fentanyl (1 ng/mL) and norfentanyl (2 ng/mL) were the same

Table 3 Comparison of oral fluid and urine testing for fentanyl using LC-QTOF-MS

		Urine		
		Detected	Undetected	Total
Oral Fluid	Detected	27	0	27
		90.0%	0.0%	90.0%
	Undetected	2	1	3
		6.7%	3.3%	10.0%
	Total	29	1	30
		96.7%	3.3%	100.0%

Overall agreement, 93.3%

Detection agreement: moderate, significant ($k = 0.47$; p value = 0.002)

Table 4 Comparison of oral fluid and urine testing for acetylfentanyl using LC-QTOF-MS

		Urine		
		Detected	Undetected	Total
Oral Fluid	Detected	4	1	5
		13.3%	3.3%	16.7%
	Undetected	5	20	25
		16.7%	66.7%	83.3%
	Total	9	21	30
		30.0%	70.0%	100.0%

Overall agreement, 80.0%

Detection agreement: moderate, significant ($k = 0.45$; p value = 0.007)

for oral fluid and urine. Both fentanyl and norfentanyl are present at much lower concentrations in oral fluid than urine; the median concentrations (ng/mL) of fentanyl and norfentanyl in oral fluid and urine were reported as 1.5 vs 13 (for fentanyl) and 1.45 vs 206 (for norfentanyl) among a group of chronic pain patients [25]. The threshold detection limit for these drugs in oral fluid will necessarily need to be lower than urine for detection in the two matrices to be equivalent. We believe that our urine testing methodology identified fentanyl in the urine of all exposed participants. One explanation for the lack of fentanyl detection in the oral fluid of two exposed individuals (as well as the overall absence of norfentanyl detection in oral fluid) is that the LODs in oral fluid were too high for this study population.

In addition to analytical considerations, the choice of matrix for drug monitoring is also dependent on patient acceptance or willingness to provide an oral fluid or urine specimen. Previous studies have demonstrated the acceptability and feasibility of using oral fluid to detect the presence of other opioids in oncology patients [26]. In our study, it was found that heroin users in the Emergency Department setting were highly accepting of oral fluid swabs for drug testing after overdose, in addition to traditional urine collection procedures.

One striking feature of this study was the detection of acetylfentanyl and U-47700 in oral fluid. Four participants had acetylfentanyl detected both matrices; one participant had acetylfentanyl detected in their oral fluid only. U-47700 was detected in paired oral fluid and urine specimens from two participants. Further work is needed to evaluate the overall agreement between oral fluid and urine for these two agents, given the small sample size.

The study dates were chosen based on IRB approval and achievement of target enrollment. We did not select the study period to correlate with a known spike in regional fentanyl seizures or deaths; however, we have seen marked increases in fentanyl-associated opioid overdoses throughout Massachusetts over the past 2 years. With respect to limitations, our results may not reflect other populations and settings outside of this region and cannot be easily generalized to other EDs with patients of different demographic distributions; however, our methodology can be employed by other institutions to establish local patterns of non-pharmaceutical fentanyl and clandestine opioid use within their population. Additionally, we were unable to standardize the timing of specimen acquisition (typically obtained within 1–4 h of patient presentation). The timing of oral fluid and urine specimens was variable; factors included avoiding interference with clinical care, waiting until the patient was able to provide informed consent, and the timing of each patient's ability to provide a urine sample. The optimal sampling period for oral fluid since the time of exposure for the clandestine opioids remains unknown.

Conclusion

In this study of individuals presenting to the ED after reported heroin overdose, a strikingly high proportion (96.7%) had detectable fentanyl in their urine. Using LC-QTOF-MS, we observed a high level of agreement (93.3%) in testing results for fentanyl between paired oral fluid and urine specimens collected from this population. These initial findings suggest that the testing strategy described in this study can be applied to oral fluid to screen for fentanyl exposure after reported heroin overdose in the ED. These findings must be verified and validated in additional studies. Rigorous testing for non-pharmaceutical fentanyl and other clandestine opioids in oral fluid is not routinely available for clinical use, and the methods described herein represent an innovation to traditional drug testing for opioids that can be used to guide surveillance and public health interventions.

Author Contributions PRC, EWB, BKL, and KMB conceived the study, designed the trial, and obtained research funding. MKG, PRC, BC, and KMB undertook recruitment of participants and managed the data including quality control. MF, AJK, and BKL designed and conducted analysis of oral fluid and urine specimens. NV conducted the statistical analyses. KMB and EWB drafted the manuscript, and all authors contributed substantially to its revision. KMB takes responsibility for the paper as a whole.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

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