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Evaluation of a lateral flow immunoassay for the detection of the synthetic opioid fentanyl

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ABSTRACT

In 2017, 47,600 overdose deaths were reported to be associated with the abuse of opioids, including prescription painkillers (e.g. oxycodone), opiates (e.g. heroin), or synthetic opioids (e.g. fentanyl) within the United States. The recent spike in the presence of synthetic opioids in lots of heroin distributed on the street present specific and significant challenges to law enforcement. Synthetic opioids are extremely toxic substances, which can easily be inhaled. This type of exposure can lead to accidental overdoses by law enforcement and other first responders answering calls involving illicit drugs containing these substances. Due to this extreme toxicity, it is important for these individuals to have tools that can be easily deployed for accurate presumptive field tests. Currently, there are only a limited number of presumptive tests available for fentanyl detection. In this study, we addressed this technology gap by evaluating newly developed lateral flow immunoassays (LFIs) designed for the detection of fentanyl and its derivatives. These LFIs were evaluated for effectiveness in different biofluid matrices, following an in vivo exposure, cross-reactivity with fentanyl analogs, and in case samples. This study demonstrates that LFIs have the potential to be used by law enforcement for the detection of synthetic opioids.

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1. Introduction

There has been a recent alarming spike in overdose deaths associated with the use of synthetic opioids; in fact, in 2017 the National Institute on Drug Abuse (NIDA) reported 29,406 overdose deaths associated with these compounds [1]. Synthetic opioids (i.e. fentanyl and derivatives) can be particularly potent, with only minute quantities capable of rapidly inducing severe respiratory depression. This aspect makes these compounds much more dangerous than natural opium derivatives, such as morphine or heroin [2–5]. The scientific literature has reported that fentanyl is approximately 50 times more potent than heroin and 100 times more potent than morphine [3,6]. Another synthetic opioid that has recently been discovered in seized batches of street heroin is carfentanil [7,8]. This compound has been reported to be approximately 10,000 times more potent than morphine [5], making it even more dangerous than fentanyl. In fact, the only approved use for carfentanil is by veterinarians as a tranquilizing agent to rapidly incapacitate large wildlife (e.g. polar bears) for examination and other similar procedures [9–12]. Due to the extreme potency of these compounds, there is little room for error when adding these substances to a production lot of street heroin leading to significant increases in the chances of an opioid-induced overdose [13].

Dealing with synthetic opioids, such as fentanyl (and derivatives), has presented special challenges to both law enforcement and first responder personnel due to their extreme toxicity. During routine duties, these personnel may accidently come into contact with heroin that has been laced with synthetic opioids leading to a dangerous situation. For example, accidental exposures leading to significant injuries and hospitalizations of law enforcement agents have been reported [14]. In response to these events, it is essential

Abbreviations: CCDC, Combat Capabilities Development Command; COTS, commercial-off-the-shelf; DART, direct analysis in real time; ECT_{50}, 50% effective concentration-time; FIBF, p-fluoroisobutyryl fentanyl; FYL, fentanyl; GC, gas chromatography; IACUC, Institutional Animal Care and Use Committee; LFI, lateral flow immunoassay; LC, liquid chromatography; LOD, limit of detection; MS, mass spectrometry; NIDA, National Institute on Drug Abuse; NIST, National Institute of Standards & Technology; PBS, phosphate buffered saline.

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to provide the law enforcement/first responder/forensic community with the tools necessary to detect these substances in a rapid and cost effective way.

Traditional detection of synthetic opioids follows a two tiered process [4,15]. The initial phase is typically rapid screening for presumptive identification; these tests usually observe functional group reactivity in a colorimetric assay or by using optical spectrometry to identify generalized chemical signatures. The goal of this phase is down-select to an illicit compound as rapidly and accurately as possible in order to make an arrest. Also, these initial findings will aid in evidence selection for laboratory-based confirmatory testing. Currently, there are only a limited number of colorimetric tests available to law enforcement or first responders. One of these tests includes the NARK II Fentanyl Reagent (Sirchie; Youngsville, NC) selling for approximately $20 for 10 tests ($2/test). These tests have many drawbacks including the use of caustic chemicals, low reliability, and lack of independent validation [16]. Recent reports have suggested that the limits of detection (LODs) for colorimetric detection assays is at least greater than 10 μg [17]. In addition, there have been reports in the popular media questioning the validity of these types of colorimetric tests [18]. Once the samples have been collected and taken to the laboratory, the second tier of analysis begins. The exacting nature of this tier of testing often utilizes chromatographic separation coupled with one or more forensic analytical chemistry techniques. Most commonly, liquid or gas chromatography (LC or GC) coupled to mass spectroscopy (MS), allows for identification of unknown substances based upon retention time and accurate mass [19,20].

The use of lateral flow immunoassays (LFIs) are widely used for the detection of specific substances in medicine [21], agriculture [22], environmental science [23], and biodefense [24]. These assays are typically paper-based matrices in which antibodies directed against compounds of interest have been incorporated. LFIs have been shown to be effective at detecting a wide variety of compounds of interest for law enforcement and first responders including illicit drugs [25], pathogens [26], chemicals [27], and substances associated with bioterrorism [24]. The main advantage of these tests is that they are capable of producing results in a short time frame (<5 min), at a minimal cost ($1–4 per test), requiring no dangerous chemicals, special laboratory equipment, or extensive training for interpreting the results. Liquid sample, typically in the form of a buffered solution containing a specific analyte, is placed on the absorbent sample pad (either by pipetting or by dipping the sample pad into the test liquid) which allows capillary action to move the liquid up the strip to the test and control lines containing the capture reagents (i.e. antibodies). LFIs are typically either sandwich or competitive assays [28]. Briefly, in sandwich assays, the sample migrates up the test strip and encounters color labeled antibodies designed to detect a specific epitope. If the target compound binds to the antibody, it will then bind to other antibodies impregnated in the test line also specific to the target. This interaction causes a colored line to form, indicating a positive result. Sandwich assays are typically used when the target molecule is large, such as human chorionic gonadotropin in pregnancy tests [28]. In competitive assays, the sample first encounters colored antibody-labeled competitive binding particles. As the sample and colored antibody- labeled competitive binding particles flow over the test line, any target analyte (i.e. fentanyl) present in the sample in excess of the concentration found in the test site will block the binding site on the capture antibody bound to the test site; therefore, preventing any binding of the colored antibody-labeled competitive particle. Thus, a positive test is indicted by no colored band in the test area. In both assay types, there is a control line containing control antibodies that bind any free color-labeled detection antibodies. The appearance of this band indicates the assay was performed properly.

In the current study, we evaluated the effectiveness of commercially available competitive LFIs designed to detect the synthetic opioid fentanyl in either urine or saliva. We determined the sensitivity of these assays as well as any cross reactivity with known fentanyl analogs (e.g. carfentanil). In addition, we evaluated urine and saliva samples from rabbits that had been exposed to fentanyl to determine if the LFIs would perform following an in vivo exposure situation. Finally, we evaluated the LFIs with four individual case samples known to contain fentanyl. Overall, our evaluation suggests that the LFIs designed to detect fentanyl could easily be adapted by either law enforcement or first responders for the field detection of this compound.

2. Materials and methods

2.1. Fentanyl-specific LFIs

Initial studies were performed using both Fentanyl Rapid Test Dipsticks (urine) and the Fentanyl Rapid Test Cassettes (oral fluid) produced by Express Diagnostics Int'l Inc. (Blue Earth, MN) [29,30]. These assays are competitive LFIs where the appearance of a single band indicates a positive result or the appearance of two bands indicates a negative result. In these studies, even the slightest appearance of a second band was considered a negative result. The manufacturer has stated that in addition to reactivity to fentanyl, the antibodies used for this LFI cross-react with the metabolite of fentanyl, norfentanyl [29,30]. In addition to commercial-off-the-shelf (COTS) fentanyl LFIs, custom order fentanyl LFIs were developed by the manufacturer that contained an increased amount of detection antibodies in the test zone and were also evaluated in this study. For these experiments, we considered the LODs for the LFIs to be defined as the fentanyl concentration which produces a positive result in 100% of the experiments. Sensitivity of the assays was considered the lowest concentration of fentanyl that yields at least a single positive result in the concentrations tested.

2.2. Test compounds

Carfentanil, cocaine, fentanyl, heroin, oxycodone, and remifentanil were purchased as analytical standards (100 μg/mL diluted in methanol) from Cerilliant Corporation (Round Rock, TX). Acetyl fentanyl, butyryl fentanyl, crotonyl fentanyl, cyclopropyl fentanyl, p-fluoroisobutyryl fentanyl (FIBF), 2-furanyl fentanyl, β-hydroxythiol fentanyl, methoxy fentanyl, methyl fentanyl, norfentanyl, and U-47700 were purchased as analytical standards (100 μg/mL diluted in methanol) from Cayman Chemical (Ann Arbor, MI). A complete list of test compounds used in this study is shown in Table 1.

Table 1
List of test compounds.

Fentanyl analogs	Metabolite
Acetyl fentanyl	Norfentanyl
Butyryl fentanyl	Related compounds
Carfentanil	Heroin
Crotonyl fentanyl	Oxycodone
p-Fluoroisobutyryl fentanyl (FIBF)	U-47700
2-furanyl fentanyl	Mixture
β-hydroxythiol fentanyl	1:10 fentanyl:heroin
Methoxy fentantyl	Other
Methyl fentanyl	Cocaine
Remifentanil	

2.3. Biofluid test matrices

Both pooled normal human urine and saliva were purchased from Innovative Research (Novi, MI). Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich (St. Louis, MO). For LOD/assay sensitivity analysis, each of the biofluid matrices were spiked with increasing concentrations of the test substances.

2.4. LFI procedures

2.4.1. Urine LFIs

The LFIs were used according to the manufacturer's recommended protocol with slight modifications [29]. Prior to use, all test materials were allowed to warm to room temperature. Stocks of increasing concentrations of fentanyl analytical standards (or other experimental compounds) were added to either pooled normal human urine or PBS. Then, a 100 μL aliquot of each test concentration was placed in a well of a 96 well plate. The test strip was then submerged (up to the line marked "MAX") into the dilutions for 10 s to 20 s. Following this, the strips were placed face up on a clean, non-porous surface (e.g. aluminum foil) under a chemical fume hood. The strips were scored for a positive or negative response 5 min–10 min following exposure to the experimental samples. Finally, images were recorded with a digital camera. An example of both a positive and negative fentanyl urine LFI strip is shown in Fig. 1.

2.4.2. Saliva LFIs

These LFIs were used at room temperature according to the manufacturer's recommended protocol with slight modifications [30]. Briefly, the LFIs were removed from the packaging and placed flat on a hard surface. Stocks of increasing concentrations of fentanyl analytical standards (or other experimental compounds) were added to either pooled normal human saliva or PBS. Then, a 120 μL aliquot of each sample was pipetted into the sample well of a test cassette. The test cassettes were scored 5 min–10 min following exposure; images of the results were recorded with a digital camera. An example of a positive and a negative fentanyl saliva LFI cassette is shown in Fig. 1.

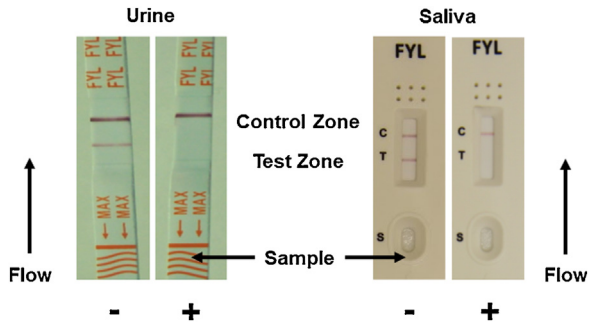

Fig. 1. Schematic of competitive urine and saliva fentanyl LFIs. A liquid sample is placed on the absorbent sample pad by either dipping (urine) or pipetting (saliva) which allows capillary action to move the liquid up the strip to the test and control lines containing the capture antibodies. Since these are competitive LFIs, the appearance of a single band in the control zone of the LFI is considered a positive result (right side LFIs), while the appearance of colored bands in both the control and test zones is considered a negative result (left side LFIs). (-) negative result; (+) positive result; C: control zone; FYL: fentanyl; LFI: lateral flow immunoassay; T: test zone; S: sample.

2.5. Experimental animal exposure

Young adult male New Zealand White rabbits, weighing 2.5 kg–2.7 kg, were procured from Covance, Inc. (Princeton, NJ) and pair-housed for 3 days before testing. Husbandry, feed/water provisions, and sanitation schedules were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* [31]. Rabbits were on a cycle of 12 h of light and 12 h of dark. Individual rabbit rooms were maintained at 21 ± 1 °C with 30% to 70% relative humidity. Rabbits were housed in a facility that was fully accredited by the Association for Assessment and Accreditation of Laboratory Care International. Rabbits were pair-housed in plastic cages on racks and provided with certified laboratory chow and reverse-osmosis water ad libitum, except during testing. Animal care and use for these experiments was approved by the Institutional Animal Care and Use Committee (IACUC) for U.S. Army Combat Capabilities Development Command (CCDC) Chemical Biological Center (Aberdeen Proving Ground, MD). Rabbit fentanyl exposures and all animal manipulations were conducted in accordance with a protocol approved by the U.S. Army CCDC Chemical Biological Center IACUC. Exposures took place for each group (n = 4) while the animals were placed in a nose-only exposure chamber, 20 cm^3 inner volume, with a flow rate of 19 L/min and under pressure of –0.5 in. H_2O. Chamber concentration was measured in real time using a TSI, Inc. Dust-Trak II, model 8530 aerosol monitor (Shoreview, MN). Two glass fiber filter pads and a 7-stage cascade impactor were used to control for chamber concentration and particle sizing, respectively. Filter pads and stages were analyzed using LC–tandem mass spectrometry (—MS) with an Agilent LC triple quadrupole 6490 system (Agilent Technologies, Inc.; Santa Clara, CA). Animal exposure durations were 5 min with 50% effective concentration-time (ECT_{50}) doses of fentanyl. Unexposed rabbits were used as controls. Euthanasia was performed in accordance with the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition* [32]. Rabbits did not have visual or auditory access to the euthanasia of other rabbits. The method of euthanasia was cervical dislocation of the C1 vertebra using a stainless steel RP-3000 rabbit and poultry wringer (MHS, LLC; West Grove, PA). The death of a rabbit was verified by three methods: loss of pupillary light response, retrobulbar reflex, and loss of respiration or cardiac arrest. Sample collection occurred immediately following the cervical dislocation procedure and samples were collected at 30 min, 4 h, and 24 h post-exposure (Fig. 2). Urine samples were collected using syringes during the necropsies. Saliva samples were collected by buccal lavage using 1 mL cold saline and suction to remove as much saliva as possible (1.2 mL to 1.4 mL). Both urine and saliva samples were centrifuged at 1000 rpm for 5 min and then stored at −80 °C until use. Urine and saliva samples from the rabbits were processed for fentanyl detection as stated above.

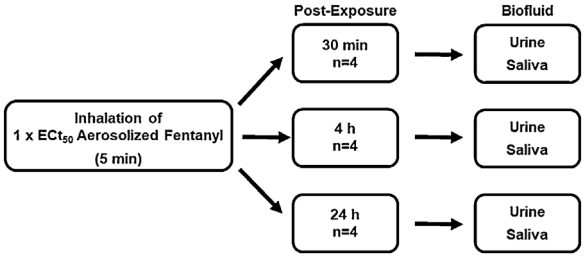

Fig. 2. Schematic of sample collection from fentanyl-exposed rabbits. Adult male New Zealand white rabbits were untreated or exposed to 1 × ECt_{50} aerosolized fentanyl for 5 min. Urine and saliva samples were collected at 30 min, 4 h, and 24 h post-exposure. A total of 28 samples were collected for analysis.

2.6. Evaluation of fentanyl analogs and related compounds

For all compounds tested [except the positive fentanyl control and the 1:10 fentanyl:heroin mixture], a 0.1 μg/mL working solution was created in PBS. The fentanyl positive control was diluted to a concentration of 2 μg/mL (or 2000 ng/mL) in PBS. The 1:10 fentanyl:heroin mixture was created by a final heroin dilution of 2 μg/mL and a final fentanyl dilution of 0.2 μg/mL. For these tests, 100 μL of the PBS-analyte solution was pipetted into the well of a 96-well plate and processed for analysis as stated above. For the saliva LFIs, 120 μL of the PBS-analyte solution was pipetted into the well of the saliva cassette and process as stated above. For additional reference, the chemical structures of the fentanyl analogs are shown in Supplemental Fig. 1. The red circles indicate modified areas of the compounds compared to fentanyl.

2.7. Case samples

Street samples from adjudicated cases were obtained from a local forensic laboratory and diluted in PBS prior to examination by the LFIs. Case #1 contained a mixture of fentanyl, heroin, and quinine; Case #2 contained a mixture of caffeine, mannitol, and fentanyl; Case #3 contained a mixture of acetyl fentanyl, fentanyl, and heroin; and Case #4 contained a mixture of alprazolam, etizolam, and fentanyl. For reference, the GC–MS chromatograph and the direct analysis in real time (DART)-MS spectra for each case sample are shown in Supplemental Fig. 2.

3. Results and discussion

3.1. Evaluation of urine-specific fentanyl LFIs

We evaluated the sensitivity and LOD for both the COTS and custom urine-specific fentanyl LFIs by spiking pooled normal human urine with increasing concentrations of analytical standard fentanyl (Table 2). The LOD for the COTS LFI was determined to be 8 ng/mL and the sensitivity to be 5 ng/mL (75%). The LOD for the custom LFI was considered 25 ng/mL and its sensitivity to be 8 ng/mL (8%).

3.2. Evaluation of saliva-specific fentanyl LFIs

The sensitivity and LODs for both the COTS and custom saliva-specific fentanyl LFIs was determined by adding increasing concentrations of analytical standard fentanyl to pooled normal human saliva (Table 3). Both the COTS and custom fentanyl LFIs detected 100% of the fentanyl-containing samples at a concentrations ≥100 ng/mL (LOD). Concentrations as low as 75 ng/mL were detected by the COTS LFIs (at a rate of 60%), while this same concentration was detected in 69% of the samples tested in the custom LFIs. Using the custom fentanyl saliva LFI, 50 ng/mL fentanyl was detected at a rate of 18% (sensitivity). This concentration was undetectable using the COTS saliva LFIs. Interestingly, we were able to increase the sensitivity and LOD of the custom LFI, by switching the test matrix from saliva to PBS. The custom LFI detected 100% of the samples containing 75 ng/mL fentanyl (LOD). Also, the sensitivity increased to 25 ng/mL fentanyl (17% positive) when the test matrix was changed from saliva to PBS.

Table 2
Analysis of urine-based fentanyl LFIs. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Concentrations with 100% detection of fentanyl are shown in bold. COTS: commercial-off-the-shelf; FYL: fentanyl; LFI: lateral flow immunoassay.

Test matrix: urine	#Positive samples/total (%)	
Fentanyl concentration (ng/mL)	COTS FYL LFI	Custom FYL LFI
2000	**14/14 (100)**	**9/9 (100)**
150	**8/8 (100)**	Not evaluated
100	**12/12 (100)**	**8/8 (100)**
75	**8/8 (100)**	Not evaluated
50	**16/16 (100)**	**12/12 (100)**
25	**12/12 (100)**	**13/13 (100)**
10	**16/16 (100)**	2/13 (15)
8	**8/8 (100)**	1/13 (8)
5	12/16 (75)	0/13 (0)
1	0/8 (0)	Not evaluated
0	0/14 (0)	0/11 (0)

Table 3
Analysis of saliva-based fentanyl LFIs. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Concentrations with 100% detection of fentanyl are shown in bold. The third column is results using PBS instead of a saliva matrix. COTS: commercial-off-the-shelf; FYL: fentanyl; LFI: lateral flow immunoassay; PBS: phosphate buffered saline.

Test matrix: saliva	#Positive samples/total (%)		
Fentanyl concentration (ng/mL)	COTS FYL LFI	Custom FYL LFI	Custom saliva LFI (PBS)
2000	**12/12 (100)**	**15/15 (100)**	**9/9 (100)**
150	**12/12 (100)**	**17/17 (100)**	**12/12 (100)**
100	**12/12 (100)**	**17/17 (100)**	**12/12 (100)**
75	9/15 (60)	11/16 (69)	**12/12 (100)**
50	0/15 (0)	3/17 (18)	8/12 (67)
25	0/15 (0)	0/17 (0)	2/12 (17)
10	0/12 (0)	0/17 (0)	0/12 (0)
5	0/8 (0)	0/14 (0)	0/12 (0)
1	0/8 (0)	0/9 (0)	Not evaluated
0	0/12 (0)	0/15 (0)	0/10 (0)

3.3. Evaluation of urine and saliva from fentanyl-exposed rabbits

In order to determine if the LFIs were capable of detecting fentanyl in biofluids following an in vivo exposure, both urine and saliva samples were collected from adult male New Zealand white rabbits following an aerosolized fentanyl exposure (ECT_{50}, 5 min). Samples were collected at 30 min, 4 h, and 24 h post-exposure as stated above. The custom fentanyl LFIs (urine/saliva) were used for these experiments. The results of these tests are shown in Table 4. Urine collected from the exposed rabbits all tested positive for the presence of fentanyl at both 30 min and 4 h post-exposure. Urine from 3 of the 4 exposed rabbits detected the presence of fentanyl 24 h following the experimental exposure. In addition, only rabbit saliva collected 30 min after the exposure tested positive (3 out of 3; saliva from one rabbit was unreadable on the LFIs). No fentanyl was detected in the rabbit saliva at either 4 or 24 h post-exposure. These results suggest that the LFIs are capable of detecting the presence of fentanyl in urine (up to 24 h) and saliva (up to 30 min) following a potential exposure (Table 4); therefore, it might be possible to use these LFIs as forensic use presumptive tests in overdose cases.

3.4. Evaluation of LFI reactivity for fentanyl analogs and other related compounds

Analogs of fentanyl have been increasingly discovered in lots of seized illegal opioids as well as during post-mortem evaluations of fatal overdoses [33–36]. Since these compounds are likely to be encountered in the field, it is important to determine if the LFIs designed to detect fentanyl will also cross-react with these compounds. To do this, a total of 11 commonly encountered fentanyl analogs were evaluated for reactivity with the custom

Table 4
Sample analysis from fentanyl exposed rabbits. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Concentrations with 100% detection of fentanyl are shown in bold. FYL: fentanyl; LFI: lateral flow immunoassay.

		#Positive samples/total (%)	
Condition	Rabbit number	Custom urine LFI	Custom saliva LFI
Unexposed	207	0/6 (0)	0/4 (0)
	208	0/6 (0)	0/4 (0)
30 min post-exposure	**195**	**6/6 (100)**	**4/4 (100)**
	196	**6/6 (100)**	Failed
	199	**6/6 (100)**	**4/4 (100)**
	200	**6/6 (100)**	**4/4 (100)**
4 h post-exposure	**197**	**6/6 (100)**	0/4 (0)
	198	**6/6 (100)**	0/4 (0)
	201	**6/6 (100)**	0/4 (0)
	202	**6/6 (100)**	0/4 (0)
24 h post-exposure	**203**	**6/6 (100)**	0/4 (0)
	204	0/6 (0)	0/4 (0)
	205	**6/6 (100)**	0/4 (0)
	206	**6/6 (100)**	0/4 (0)
Human urine (2 μg/mL FYL)		**6/6 (100)**	
Human urine		0/6 (0)	
Human saliva (2 μg/mL FYL)			**4/4 (100)**
Human saliva			0/4 (0)

urine and saliva fentanyl tests at a concentration of 100 ng/mL (Table 5). Five of the 11 fentanyl analogs evaluated tested positive in 100% of the tests (acetyl fentanyl, cyclopropyl fentanyl, β-hydroxythiol fentanyl, methyl fentanyl, and norfentanyl) at this concentration in either LFI form factor. Butyryl fentanyl, crotonyl fentanyl, p-FIBF, and 2-furanyl fentanyl displayed positive results in ~40% to 60% of the tests. Carfentanil, methoxy fentanyl, and remifentanil were undetectable with these LFIs. Additional tests were performed to determine if these compounds were detectable at increased concentrations compared to the initial evaluations (Table 6). Increasing the concentrations of butyryl fentanyl, crotonyl fentanyl, *p*-FIBF, and 2-furanyl fentanyl to 0.5 μg/mL caused these compounds to be detected in 100% of the LFIs tested (urine- or saliva-based LFIs). Both carfentanil (10 μg/mL) and methoxy fentanyl (1 μg/mL) were still undetectable with increased concentration tests. In addition to fentanyl analogs, it is also important to evaluate these LFIs for the potential cross-reactivity with other compounds of interest, such as heroin, oxycodone, U-47700, and cocaine (Table 7). These compounds produced negative results in both the urine- and saliva-based LFIs at concentrations of 100 ng/mL. In addition, a 1:10 mixture of fentanyl (100 ng/mL) and heroin (1 μg/mL) was evaluated for LFI reactivity; all tests produced positive results. Even though the LFIs failed to detect carfentanil, methoxy fentanyl, and remifentanil it is important to note that these LFIs did detect 8 of the 11 fentanyl analogs examined (Tables 5–7). This gives a large amount of coverage of the fentanyl analogs that could be encountered during a drug arrest involving opioids.

Table 5
Analysis of fentanyl analogs. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Compounds in bold represent 100% detection of the fentanyl analog. LFI: lateral flow immunoassay.

Test matrix: PBS	#Positive samples/total (%)	
Fentanyl analog (0.1 μg/mL)	Custom urine LFI	Custom saliva LFI
Acetyl fentanyl	**7/7 (100)**	**7/7 (100)**
Butyryl fentanyl	3/7 (43)	2/7 (29)
Carfentanil	0/4 (0)	0/4 (0)
Crotonyl fentanyl	3/7 (43)	2/7 (29)
Cyclopropyl fentanyl	**7/7 (100)**	**7/7 (100)**
p-Fluoroisobutyryl fentanyl (FIBF)	4/7 (57)	1/7 (14)
2-Furanyl Fentanyl	4/7 (57)	4/7 (57)
β-Hydroxythiol fentanyl	**7/7 (100)**	**7/7 (100)**
Methoxy fentanyl	0/7 (0)	0/7 (0)
Methyl fentanyl	**7/7 (100)**	**7/7 (100)**
Norfentanyl	**7/7 (100)**	**7/7 (100)**
Remifentanil	0/4 (0)	0/4 (0)

Table 6
Analysis of increased concentration of fentanyl analogs. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Compounds in bold represent 100% detection of the fentanyl analog. LFI: lateral flow immunoassay; PBS: phosphate buffered saline.

Test matrix: PBS	#Positive samples/total (%)	
Test compounds	Custom urine LFI	Custom saliva LFI
Fentanyl (2 μg/mL)	**9/9 (100)**	**9/9 (100)**
Butyryl fentanyl (0.5 μg/mL)	**4/4 (100)**	**1/1 (100)**
Crotonyl fentanyl (0.5 μg/mL)	**4/4 (100)**	**1/1 (100)**
p-Fluoroisobutyryl fentanyl (0.5 μg/mL)	**4/4 (100)**	**1/1 (100)**
2-Furanyl fentanyl (0.5 μg/mL)	**4/4 (100)**	**1/1 (100)**
Methoxy fentanyl (1 μg/mL)	0/4 (0)	0/1 (0)
Carfentanil (10 μg/mL)	0/4 (0)	0/1 (0)

Table 7
Analysis of other compounds of interest. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Compounds in bold represent 100% detection of the fentanyl analog. LFI: lateral flow immunoassay; PBS: phosphate buffered saline.

Test matrix: PBS	#Positive samples/total (%)	
Other compounds (0.1 μg/mL)	Custom urine LFI	Custom saliva LFI
Fentanyl	**8/8 (100)**	**7/7 (100)**
Heroin	0/7 (0)	0/7 (0)
Oxycodone	0/7 (0)	0/7 (0)
U-47700	0/7 (0)	0/7 (0)
1:10 fentanyl:heroin mixture	**7/7 (100)**	**7/7 (100)**
Cocaine	0/7 (0)	0/7 (0)
PBS	0/8 (0)	0/8 (0)

3.5. Evaluation of fentanyl-containing case samples

Fentanyl is almost never found in pure form in street drugs; it is almost always found as a mixture with other illegal substances (e.g. heroin) as well as cutting agents or diluents. Since this is the case, it is important to determine if the fentanyl LFIs are capable of detecting the presence of fentanyl in samples that could likely be encountered in the field. To do this, four fentanyl-containing case samples previously seized by law enforcement were examined. In these studies, each case sample was tested against 6 custom urine-specific LFIs. All LFIs displayed a single band in the control zone, therefore, indicating positive results for fentanyl in each of the case samples examined (Table 8). These results indicate that the LFIs used for this study are capable of detecting fentanyl in actual case samples.

Table 8
Analysis of case samples. Tests were performed using the custom urine fentanyl LFIs. Results are reported as the number of positive samples over the total number of samples. The percentage of positive results are shown in parenthesis. Compounds in bold represent 100% detection of the fentanyl analog. LFI: lateral flow immunoassay.

Case#	Contents	#Positive samples/total (%)
1	Fentanyl, Heroin, Quinine	**6/6 (100)**
2	Caffeine, Mannitol, Fentanyl	**6/6 (100)**
3	Acetyl Fentanyl, Fentanyl, Heroin	**6/6 (100)**
4	Alprazolam, Etizolam, Fentanyl	**6/6 (100)**

4. Conclusions

In this study, we evaluated LFIs developed by Express Diagnostics Int'l, Inc. for the detection of the synthetic opioid fentanyl in urine, saliva, and PBS [29,30]. The LOD for the COTS versions of the urine- and saliva-based LFIs were 8 and 100 ng/mL, respectively. The sensitivity of these LFIs were determined to be 5 ng/mL (urine) and 75 ng/mL (saliva). The custom urine LFI had different LODs and sensitivities compared to the COTS versions. We identified the LOD to be 25 ng/mL and the sensitivity to be 8 ng/mL (Table 2). For the custom saliva LFIs, the LOD was determined to be 100 ng/mL when fentanyl was diluted in saliva and 75 ng/mL when diluted in PBS (Table 3). The sensitivities increased in the custom saliva LFIs. The sensitivity of these assays were 50 ng/mL for saliva and 25 ng/mL for PBS. In addition to laboratory spiked samples, the custom urine LFIs detected fentanyl in rabbit urine up to 24 h following a fentanyl exposure (Table 4). Saliva samples from these same rabbits only detected fentanyl 30 min following the laboratory exposure, suggesting only a short window of opportunity for fentanyl detection within the saliva. We also demonstrated that several fentanyl analogs could be detected using these LFIs (acetyl fentanyl, butyryl fentanyl, crotonyl fentanyl cyclopropyl fentanyl, p-FIBF, 2-furanyl fentanyl, and β-hydroxythiol fentanyl) (Tables 5,6). Finally, we demonstrated that these LFIs could detect the presence of fentanyl in actual case samples known to contain fentanyl (Table 8). In conclusion, these data associated with the fentanyl-directed LFIs have demonstrated the potential to be used in the field by law enforcement officers as presumptive fentanyl tests.

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Declaration of interest

None.

Disclaimer

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CRediT authorship contribution statement

Daniel J. Angelini: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Tracey D. Biggs:** Data curation, Writing - review & editing. **Michele N. Maughan:** Data curation, Project administration, Writing - review & editing. **Michael G. Feasel:** Data curation, Writing - review & editing. **Edward Sisco:** Data curation, Methodology, Resources, Writing - review & editing. **Jennifer W. Sekowski:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.forsciint.2019.04.019.

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