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RESEARCH ARTICLE

Simultaneous measurement of fentanyl, fentanyl analogues and other drugs of abuse by multiplex bead assay

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ABSTRACT

Quantification of illicit drugs and controlled substances, in urine or as surface contamination, is often performed using expensive analytical techniques such as liquid chromatography with tandem mass spectrometry (LC-MS/MS). A time and cost-effective semi-quantitative surface-wipe and urine screening multiplex immunoassay for fentanyl and its analogues was developed in this investigation. We previously created a surface wipe multiplex immunoassay for methamphetamine, caffeine, cocaine, tetrahydrocannabinol (THC) and oxycodone. This fluorescent covalent microsphere immunosorbent assay (FCMIA) is a competitive assay where drugs compete with protein-drug conjugates attached to microspheres for antibodies. It was assembled using a commercially available fentanyl antibody and protein-conjugate. Surface recovery from ceramic tiles was assessed by FCMIA, with results ranging from 26% for fentanyl to 60% for methamphetamine. Only fentanyl and its structurally similar analogues showed significant response to the fentanyl assay whereas, analogues structurally similar to carfentanil gave no response. Non-fentanyl drug assays did not appreciably detect fentanyl or its analogues. Overall, this method is a useful tool for assessing surface contamination and the effectiveness of decontamination by multiple drugs of abuse, potentially lowering workplace exposures. To broaden applicability, different antibodies or aptamers must be developed to detect structural differences found in classes of analogues such as carfentanil.

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Introduction

Fentanyl is a potent synthetic opioid prescribed for pain management that in recent years, has become a drug of misuse (DEA 2024). Fentanyl and its analogues, such as acetyl fentanyl, carfentanil, sufentanil, and alfentanil, quickly depress respiration, lower the heart rate, and can lead to loss of consciousness and death (NDEWS 2015). Increasing clandestine manufacturing and use of fentanyl and its analogues put public safety workers at risk for exposure. Law enforcement officers seize illicitly manufactured fentanyl analogues sold as powders, nasal sprays, or pills. According to the Centers for Disease Control and Prevention (CDC) and the National Forensic Laboratory Information System (NFLIS), the number of fentanyl seizures increased by 259% between 2013 to 2014, and fentanyl encounters rose to 14,440 (NFLIS 2021, CDC 2015). By 2022, the Drug Enforcement Administration (DEA) announced that it had seized over 50.6 million fentanyl-laced, fake prescription pills and more than 10,000 pounds of fentanyl powder over the course of that year (DEA 2022). Sixty-three exhibits analyzed by federal, state, and local forensic laboratories identified acetyl fentanyl in 2014, and this rose dramatically to 4,789 exhibits by 2020 (DEA 2023). Carfentanil, used in veterinary medicine as a tranquilizing agent and 100 times more potent than fentanyl, is also being diverted from animal hospitals. Law enforcement, emergency response personnel, laboratorians, environmental health, and remediation workers may be unknowingly exposed to opioids that interfere with their ability to perform their normal duties (Howard and Hornsby-Myers 2018; Chiu et al. 2019; NIOSH et al. 2021).

Complicating matters further, some drug dealers combine fentanyl and/or analogues with other illicit drugs including methamphetamine, cocaine, and heroin, unbeknownst to the buyer nor responding public safety personnel (Wilson et al. 2020). In 2013, of the 881 samples submitted to federal, state, and local laboratories that contained fentanyl, 77% also contained heroin, 13% also contained cocaine, and 2% also contained methamphetamines (DEA 2023). By 2017, out of 24,201 fentanyl and other mixed drug samples that were submitted, 80% contained heroin, 9% contained cocaine, 4% contained furanyl fentanyl, 4% contained carfentanil, and 4% acetyl fentanyl (DEA 2023). This increase in the variety of drugs in samples led to requests from responders and the forensic community for more tools and methods to identify

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mixtures of these substances on objects and surfaces. While the gold standard for drug identification remains to be chromatography methods paired with mass spectrometry, these methods may be cost-prohibitive for smaller precincts and laboratories.

The objective of this study was to explore a lower cost multiplexed fluorescent covalent microbead immunoassay (FCMIA) method, that can identify up to 100 analytes simultaneously through dually stained microspheres (Vignali2000) for illicit drug detection from surface wipe samples. These assays are relatively inexpensive, can be run in a day, and have high throughputs (Biagini et al. 2004). In the past, we have developed FCMIA methods to identify multiple pesticides in water and urine (Biagini et al. 2004) as well as drugs of abuse such as cocaine, tetrahydrocannabinol (THC), oxycodone, heroin, and methamphetamine in water and urine, and on surfaces as described by Smith et al. (2010). In the latter study, we reported a least detectable dose (LDD) of 0.37, 0.36, 0.22, 0.49 and 0.06 ng/mL respectively. In this study, we investigated if a fentanyl assay could be included with the previous assays in FCMIA to measure a combination of drugs on surfaces, including fentanyl analogues (Figure 1).

Materials and methods

Chemicals

Standard solutions for fentanyl (1 mg/mL in methanol), alfentanil (1 mg/mL in methanol), butyryl fentanyl (100 µg/mL in methanol), carfentanil (100 µg/mL in methanol), furanyl fentanyl (100 µg/mL in methanol), remifentanil (100 µg/mL in methanol), sufentanil (100 µg/mL in methanol), heroin (1 mg/mL in acetonitrile), tetrahydrocannabinol (THC) (100 µg/mL in methanol), methamphetamine (1 mg/mL in methanol), and cocaine (1 mg/mL in acetone) were obtained from Cerilliant Corporation (Round Rock, TX). The assay requires drug-protein conjugates to be coupled to microspheres, and in this case the drugs were conjugated to bovine serum albumin (BSA). Pre-conjugated methamphetamine-BSA (AGMET-0300), benzoylecgonine-BSA (AGCOC-0307), tetrahydrocannabinol-BSA (AGTHC-0306), and morphine-BSA (AGMOR-0310) were obtained from Arista Biologicals (Allentown, PA). Monoclonal antibodies to methamphetamine (ABMET-0400), benzoylecgonine (ABCOC-0402), tetrahydrocannabinol (ABTHC-0401), and morphine (ABMOR-0401) were also obtained from Arista Biologicals. Fentanyl-BSA (MBS5304704) and the fentanyl monoclonal antibody

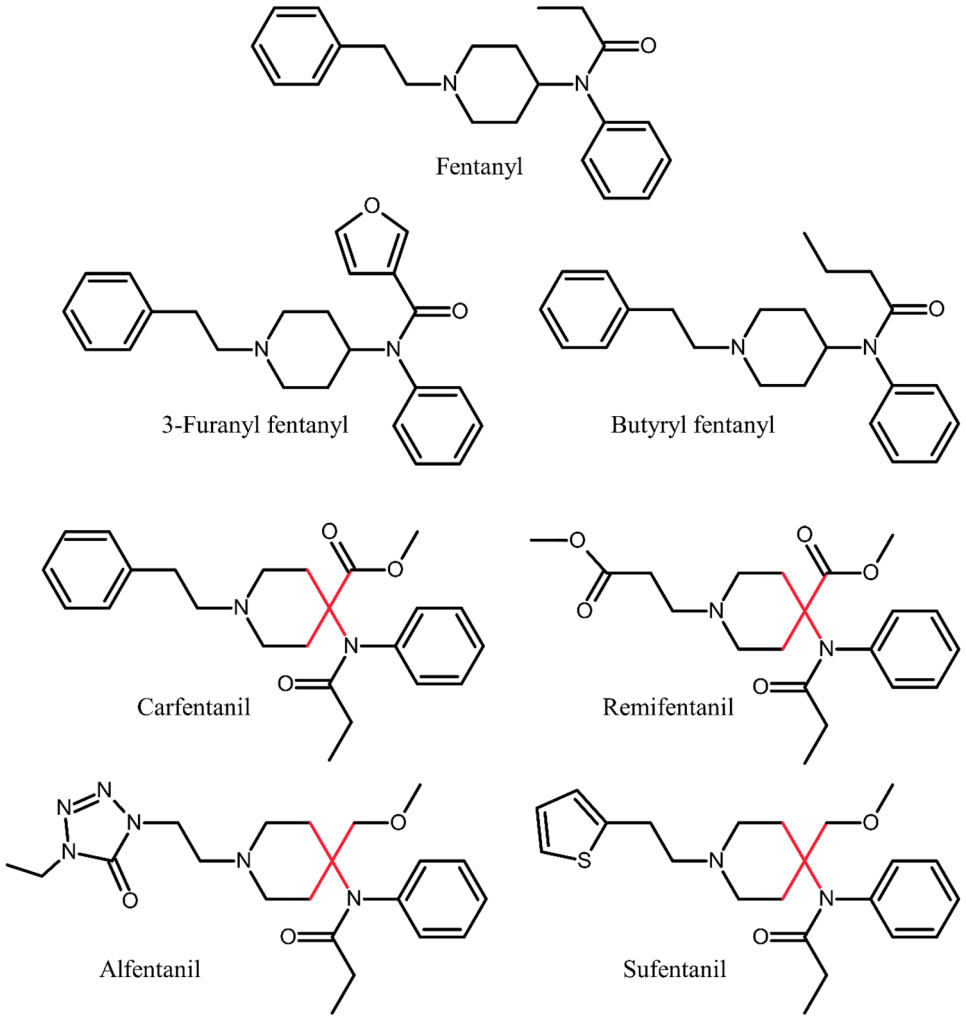

Figure 1. Structures of fentanyl and its analogues. Note that butyryl fentanyl and furanyl fentanyl have the same basic structure as fentanyl, while alfentanil, remifentanil, and sufentanil have similarities to carfentanil (red X).

(MBS838396) were obtained from MyBioSource (San Diego, CA). Deionized type I water (18 MΩ-cm), produced by a Milli-Q® Integral 15 water purification system from Millipore Sigma (Burlington, MA), was used for all procedures. MagPlex® magnetic microspheres (beads) were obtained from Luminex Corporation (Austin, TX). The activation buffer used was 0.1 M monosodium phosphate (NaH_2PO_4), pH 6.2 and the coupling buffer was 0.05 M 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.0. Phosphate buffered saline (PBS) composed of 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH 7.4) was made using prepared packets (Sigma-Aldrich, St. Louis, MO) for sampling and extraction. PBS was selected because it had been utilized in sampling surfaces for another fentanyl screening method, a lateral flow immunoassay that is in development. Storage/blocking buffer was PBS-TBN (PBS containing 0.1% BSA, 0.02% Tween-20 and 0.05% NaN_3). Biotin-labeled goat anti-mouse IgG, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Streptavidin R-phycoerythrin (streptavidin R-PE) was obtained from Molecular Probes (Eugene, OR).

Equipment

An eight-tube magnetic tube separator was used for microsphere preparation (Pureproteome™ magnetic stand; Sigma Aldrich, St Louis MO). Assay bead wash steps were performed using a handheld magnetic separator block for 96 well flat bottom plates or 96 well conical well plates (EMD Millipore, Billerica, MA). Microtiter plates were black-clear flat bottom polystyrene #3651 (Corning Inc, Corning NY). Transfer pipettes for bead preparation were 1 mL polypropylene extended fine tip #231 (Samco/Fisher Waltham, MA). Mixtures were suspended using an ultrasonic bath (Cole-Parmer, Vernon Hills, IL), and mixed using a vortex shaker (Vortex Genie VWR, Intl., West Chester, PA). A Barnstead Labquake shaker rotisserie from Barnstead International (Dubuque, IA) was used to mix protein, buffer, and beads in the coupling reaction. A Stat Fax® 2200 shaker/incubator from BIO-RAD Corporation (Hercules, CA) was utilized to mix and incubate microtiter assay plates. A Luna-II™ (Logos Biosystems, Annandale, VA) cell counter was used to determine the concentration of coupled beads. Analyses were performed on a MagPix® instrument (Luminex Corp, Austin TX) with xPONENT® operating software.

Tiles used for surface recovery spiking study were 10 cm × 10 cm (100 cm^2) ceramic bathroom tiles. The swabs for sampling the tiles were cotton tipped with wooden handles (Puritan 806-WC; Puritan, Guilford, ME).

Preparation of microspheres

The FMCIA consists of multiple individual assays occurring simultaneously within a mixture in the same well. It takes advantage of microsphere sets that can be coupled to biomolecules such as drug-protein conjugates and can be selectively identified in a mixture. This assay used magnetic 6.5 µm MagPlex® microspheres (Luminex Corp, Austin, TX) composed of polystyrene, divinyl benzene, and methacrylic acid, providing surface carboxylate functionality for covalent attachment of biomolecules. Internally, they are fluorescently dyed in spectrally distinct regions, allowing the MagPix® analyzing instrument to recognize the different microsphere sets in a single well. The instrument contains a magnetic imaging chamber where microspheres are held in place while red LEDs at 635 nm wavelength excite internal bead dyes and green LEDs at 525 nm excite the captured reporter streptavidin R-Phycoerythrin (R-PE) reporter attached to their surfaces. The imager sorts the bead signals by the analyte to which it was coupled and the corresponding streptavidin R-PE reporter signal, thus measuring the analyte concentration.Five unique sets of carboxylate-modified magnetic microspheres were selected to be coupled with commercially procured protein-drug conjugates. Microsphere stock solutions at 1.25×10^7 microspheres/mL were aliquoted (80 µL) into 5 separate 1.5 mL centrifuge tubes to obtain 10^6 microspheres for each microsphere set. The centrifuge tubes were then placed into the magnetic separator for 2 min, forming a pellet. The liquid portion was removed, while keeping the centrifuge tubes in the magnetic separator, using a 1 mL transfer pipette and being careful not to disturb the pellet. Activation buffer (80 µL) was added to each centrifuge tube and vortexed. The centrifuge tubes were then placed into the magnetic separator for 2 min and the liquid was removed as described above. This wash step with activation buffer was repeated one more time. Next, the beads were resuspended in 80 µL of activation buffer, vortexed gently and sonicated. Ten µL of 50 mg/mL sulfo-NHS in activation buffer was added to the mixture followed by 10 µL of 50 mg/mL EDC in activation buffer. The mixture was allowed to sit for 20 min 22° $C \pm 2°$.

The tubes were placed in the magnetic separator, and the liquid was removed as described above. Each tube was washed two more times with 500 µL coupling buffer, then, resuspended in 500 µL coupling buffer and vortexed gently. Protein-drug conjugates (5 µg) were dissolved in 500 µL coupling buffer and added to each tube of selected activated microspheres. The resulting 1000 µL mixture was incubated for 2 h at 22° $C \pm 2°$, with gentle motion on a tube rotator. The coupled microspheres were then washed twice in 1000 µL storage/blocking buffer. Microsphere concentrations were determined by using a cell counter. Typically, between 10^6 and 1.25×10^7 microspheres were coupled per batch for all analytes. Coupled microspheres were stored at 4 °C ± 2° in 500 µL storage/blocking buffer.

Multiplexed assay

The multiplexed assay is a competitive assay, where analyte in solution competes with a microsphere bound drug-BSA conjugate for an anti-analyte antibody. This results in less antibody being bound to the microsphere when higher drug concentrations are present. The anti-analyte antibody bound to the microsphere is detected with a labeled secondary antibody (biotin labeled anti-mouse IgG) which in turn binds the fluorescent label (streptavidin R-PE). Therefore, streptavidin R-PE fluorescent signal from the microsphere decreases with increasing drug concentration. The fluorescent signal from

the microsphere sets is measured as mean fluorescence intensity (MFI) for each microsphere set and typically several hundred microspheres from each set are measured to obtain this mean value. A minimum of 50 microspheres from each microsphere set is needed for the MFI to be valid.

Standard mixtures of heroin, THC, methamphetamine, and fentanyl were prepared at eight concentrations (15, 7.5, 3.75, 1.88, 0.94, 0.46, 0.23, and 0 ng/mL) in 0.01 M PBS and cocaine was prepared at 5000, 2500, 1250, 625, 312, 156, 78 and 0 ng/mL. Solutions of alfentanil, butyryl fentanyl, carfentanil, furanyl fentanyl, remifentanil, and sufentanil were also prepared at eight concentrations (15, 7.5, 3.75, 1.88, 0.94, 0.46, 0.23, and 0 ng/mL). In addition, solutions of fentanyl analogues were prepared at 0, 10, 100, 1000 ng/mL to study cross-reactivity. These analogues (Figure 1) were chosen due to their prominence on DEA reports at the time of experimentation (NDEWS 2015).

The five analyte-conjugated microsphere set solutions were prepared at a working concentration of 100 microspheres/µL in storage/blocking buffer. This microsphere solution was added (25 µL) to the wells of a microtiter plate. Next, drug-analyte standards or extract solutions (50 µL) were added to the microspheres in the microtiter plate. The primary antibody mixture was prepared in storage/blocking buffer (anti-methamphetamine-0.1 µg/mL; anti-fentanyl-0.02 µg/mL; anti-benzoylecgonine-0.02 µg/mL; anti-THC-0.3 µg/mL; and anti-morphine-0.15 µg/mL). The primary antibody solution (25 µL) was added to all wells. This mixture of analytes, microspheres and antibodies was allowed to incubate at 25° $C \pm 2°$ (protected from light) for 30 min on the microplate shaker at 675 rpm. All subsequent incubations were performed in darkness.

The wells were washed twice with storage/blocking buffer using the microtiter plate magnetic separator. Biotin labeled anti-mouse IgG prepared in storage/blocking buffer at a concentration of 5 µg/mL was added (50 µL) to all wells and incubated at 25° $C \pm 2°$ for 30 min on the microplate shaker. The wells were again washed with storage/blocking buffer twice. Streptavidin R-PE reporter, prepared at a concentration of 4 µg/mL in storage/blocking buffer was added (50 µL) to all wells, and the mixture was again allowed to incubate on a microplate shaker at 25° $C \pm 2°$ for 30 min. Finally, the wells were again washed twice with storage/blocking buffer.

The microspheres were resuspended in 100 µL of storage/blocking buffer and shaken vigorously at 1500 rpm for 2 min to disperse the microspheres. The plate was placed into the autosampler platform of the MagPix®. Software provided by the manufacturer was used to gather data from the microspheres. The instrument was set up using calibration and verification microspheres and drive fluid from the manufacturer. The instrument was programmed to collect data from a minimum of 50 microspheres for each analyte (classified by their internal fluorescence ratio) and acquire the MFI of the microsphere antibody complex with a fluorescent reporter (microsphere- (fentanyl-BSA conjugate)-(primary mouse anti-fentanyl IgG antibody)-(secondary biotinylated anti-mouse IgG antibody)-(streptavidin-phycoerythrin reporter)) complex as analyzed by the MagPix software.

Spiking and sampling tiles

Tiles were spiked to produce surface loadings of 0–45 ng/100 cm^2 for fentanyl, heroin, THC, and methamphetamine and 0–7500 ng/100 cm^2 for cocaine. Using a pipettor, 50 µL of spiking solution was placed on each tile surface. Next, with the pipettor plunger still depressed, the tip was used to spread the solution across the tile using a spiral motion. The loaded tile was allowed to dry for 30 ± 5 min and then sampled with a swab wetted in PBS. The wiping pattern across the tile consisted of three passes; (1) an up and down direction or W pattern, (2) a right to left pattern or Z pattern and (3) the up and down pattern repeated as shown in Figure 2. The wiping was done with overlapping strokes such that the entire area of the tile was sampled. The swab was then put into a vial with 1 mL of extraction buffer. The cotton tip was broken off and the cap replaced. The swab was then extracted for 3 min with vigorous manual shaking. The cotton tip was removed. The tile extracts were frozen at −60° $C \pm 10°$ until analysis and thawed to 22° $C \pm 2°$for analysis. The experiment was repeated with a total of six tiles spiked at each concentration level.

Data analyses

Standard solutions

For prepared standard solutions, curves were constructed from four-parameter logistic-log fits (4-PL; SigmaPlot, SPSS,

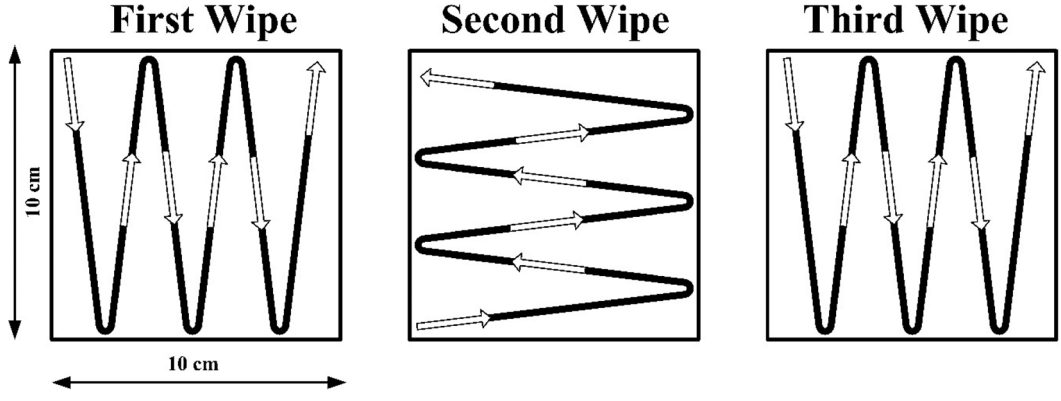

Figure 2. Wiping pattern for spiked tiles: Tiles were spiked and wiped using a three-pass method—W, Z, W.

Chicago, IL). This fit uses the ratio of signal where antibody is bound to antigen in a sample (Bs) to the signal where no antigen is present (Bs_o); thus, $\%Bs/Bs_o$ is where Bs = MFI response of drug in standard solution at a given concentration and Bs_o is MFI response to the blank solution. The least detectable dose (LDD) can be defined as the amount of an analyte that inhibits 10% of the antibody binding in an assay (Aspland and Hennion 1997). Thus, the LDD was defined as 90% Bs/Bs_o (Lawruk et al. 1993; Hennion and Barcelo 1998; Kobusińska et al. 2020) and was interpolated mathematically from the coefficients of the best-fit (4-PL) equation. Results from calibration curves run in duplicate on three different days were used to calculate the LDD. Table 1 shows LDD calculations based on 90% Bs/Bs_o. Additionally, LDD was calculated using the standard deviations of blank standards. The standard deviations were multiplied by three and subtracted from the mean blank signal (Schwalbe et al. 1984). Assessment of the 'goodness of fit' and the dynamic ranges of the assays were investigated by evaluating the fit of the standards data to the 4-PL model by 'standards recovery' (Nix and Wild 2001), calculated by evaluating interpolated results from each 4-PL fit (observed concentration) and comparing it to the concentrations of drugs added to the system (expected concentration) using the following equation:

$$\%Recovery = \frac{\text{Observed concentration}}{\text{Expected concentration}} \times 100$$

The resultant data were analyzed in the linear range of the assay (0.23–15 ng/mL for fentanyl, heroin, THC, and methamphetamine and 78–5000 ng/mL for cocaine) *via* linear regression.

Surface recovery from spiked tiles

Recovery from the tiles was calculated by determining the concentration of the solution from sampling the tiles at each surface loading (including the blank tile) using the curve from the prepared standard solutions. Thus, $\%Bt/Bs_o$ (Bt = MFI response for the solutions from sampling tiles at different loadings and Bs_o = MFI response to the blank standard solution was calculated for the solutions at each surface loading including the blank tile and the parameters of the 4-PL fit for the standard curve were used to back calculate the concentration of the solution from the sampled tile. This was compared to actual loading by linear regression to assess recovery.

Results

Standard curves

Figure 3 Panel A shows the response of the assay for fentanyl, heroin, THC, and methamphetamine and Panel B shows the assay response to cocaine in terms of $\%Bs/Bs_o$. All the drugs except for cocaine show response over the range 0–15 ng/mL while the cocaine response is over the range 0–5000 ng/mL. The THC assay can only be used in the range 0–3.75 ng/mL since the 7.5 and 15 ng/mL standards are outside of the range of the assay ($\%Bs/Bs_o$ < 1%). The methamphetamine assay is slightly less sensitive than the other drugs shown in Figure 3 Panel A. These $\%Bs/Bs_o$ curves were fitted with 4-PL model and the 4-PL parameters were used to evaluate the goodness of fit using the back-calculation of the standards (standards recovery). Figure 4 Panels A-D shows standards recovery for all drugs in the multiplex assay except cocaine and Panel E shows the same data for cocaine. All drugs show good fit by standards recovery.

Table 1. Least detectable dose calculated by 90% Bs/Bs_o and using 3 standard deviations subtracted from the blank standard.

	LDD ng/mL (90% Bs/Bs_o)	LDD ng/mL (3 × SD)
Fentanyl	0.07	0.16
Heroin	0.11	0.19
THC	0.08	0.24
Methamphetamine	0.41	0.75
Cocaine	57.7	43.7

Surface recovery from spiked tiles

Surface recovery was calculated from the concentration of the solutions from tile sampling at various loadings including the blank using the curves from the prepared standard solutions. For each drug, the recovered concentration was determined at each loading with the blank tile subtracted from each value. Using the equation above percent recovery from tiles was calculated by dividing the concentration found on a wiped tile by the expected concentration and multiplying by 100. Table 2 shows the recovery of drugs from the surfaces of spiked tiles.

Response to fentanyl analogues

Figure 5 Panels A-F shows the response of the fentanyl assay to alfentanil, butyryl fentanyl, carfentanil, furanyl fentanyl, remifentanil, and sufentanil over the range 0–15 ng/mL for each analogue. Only butyryl fentanyl and furanyl fentanyl show measurable response in the fentanyl assay. Butyryl fentanyl gave 40% the response of fentanyl and furanyl fentanyl gave greater than 100%. none of the analogues showed significant response in the heroin, THC, methamphetamine, or cocaine assays.

Response to fentanyl analogues at higher concentrations

To investigate whether the drug assays had response to higher concentrations of the analogues, the response was investigated at 10, 100, and 1000 ng/mL. The results from the fentanyl assay are shown in Figure 6 where the fentanyl assay $\%Bs/Bs_o$ for each analogue is plotted against the analogue concentration. The responses of butyryl fentanyl and furanyl fentanyl were outside the range of the fentanyl assay at 100 ng/mL and 1000 ng/mL. Both analogues gave response near the upper range of the assay at 10 ng/mL. This is expected based on the results from the response experiments at 0–15 ng/mL analogue concentration. Alfentanil, carfentanil, remifentanil and sufentanil had responses near the limit of detection in the fentanyl assay. However, remifentanil gave 50% Bs/Bs_o at 1000 ng/mL. This response is equivalent to 0.25 ng/mL fentanyl. The heroin assay showed response above the limit of detection for 1000 ng/mL of

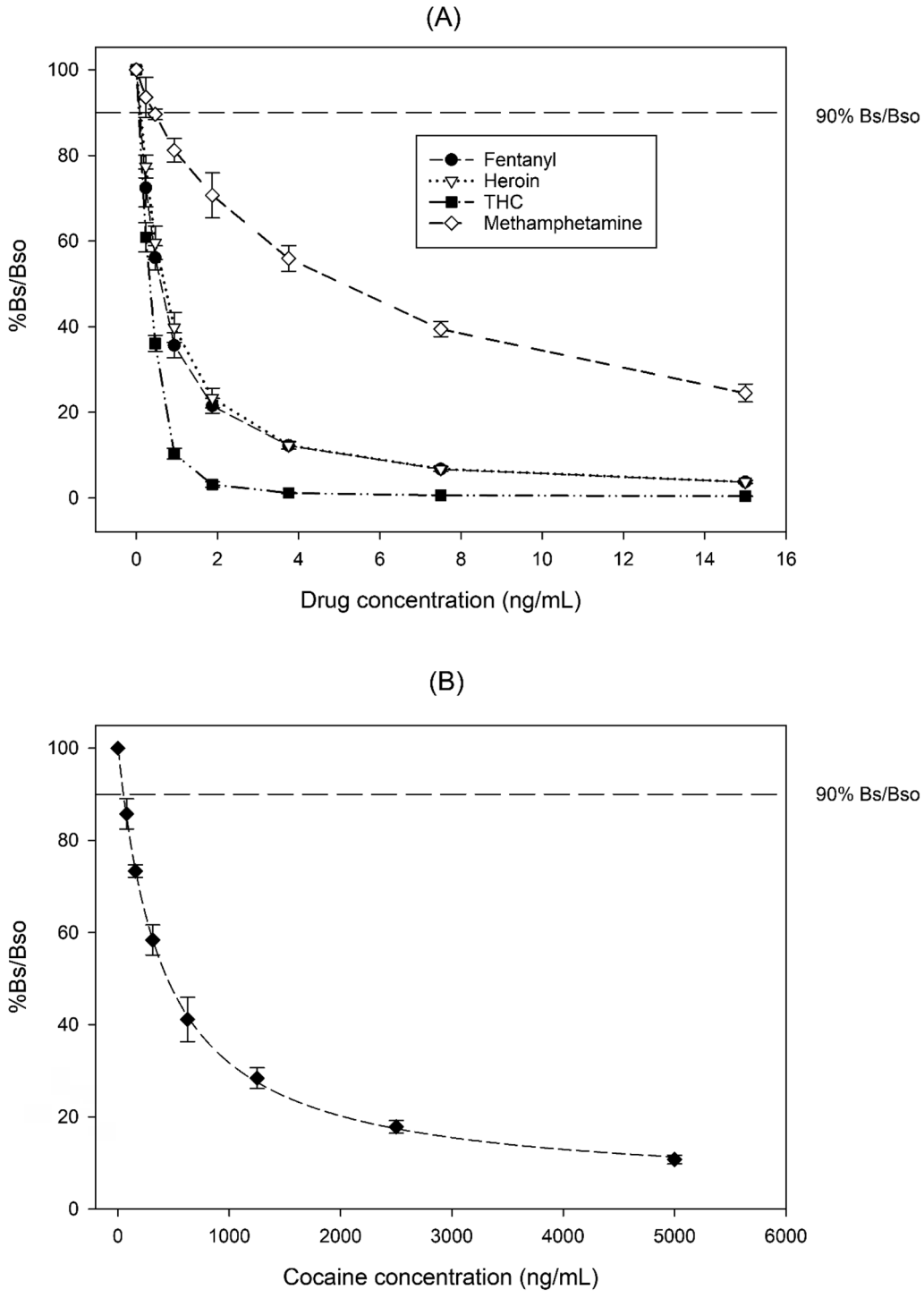

Figure 3. Drug response curves. Panel A: Response of multidrug assay to fentanyl, heroin, THC, and methamphetamine as $\%Bs/Bs_o$ over the range 0–15 ng/mL Panel B: Response of multidrug assay to cocaine as $\%Bs/Bs_o$ over the range of 0–5000 ng/mL (where Bs = MFI for each individual drug standard and Bs_o = MFI of the blank. The error bars show the standard deviation of six trials at each concentration.

butyryl fentanyl, furanyl fentanyl and sufentanil but the response is less than 0.5 ng/mL fentanyl. All other assays showed fentanyl analogue response at or below the limit of detection for the assays.

Discussion

Public safety workers exposure to drugs of abuse from surface contamination can occur during evidence handling and storage as well as when responding to calls or making entry. Methods to determine surface contamination are necessary to protect first responders and laboratory personnel. This paper describes a multidrug assay based on FCMIA that is capable of simultaneous detection and semi-quantitative measurement of five drugs of abuse (fentanyl, heroin, cocaine, THC, and methamphetamine) with LDDs less than 1 ng/100 cm^2 for all drugs except cocaine whose LDD is about 1 ng/100 cm^2. The sampling technique is simple, and the assay is capable of

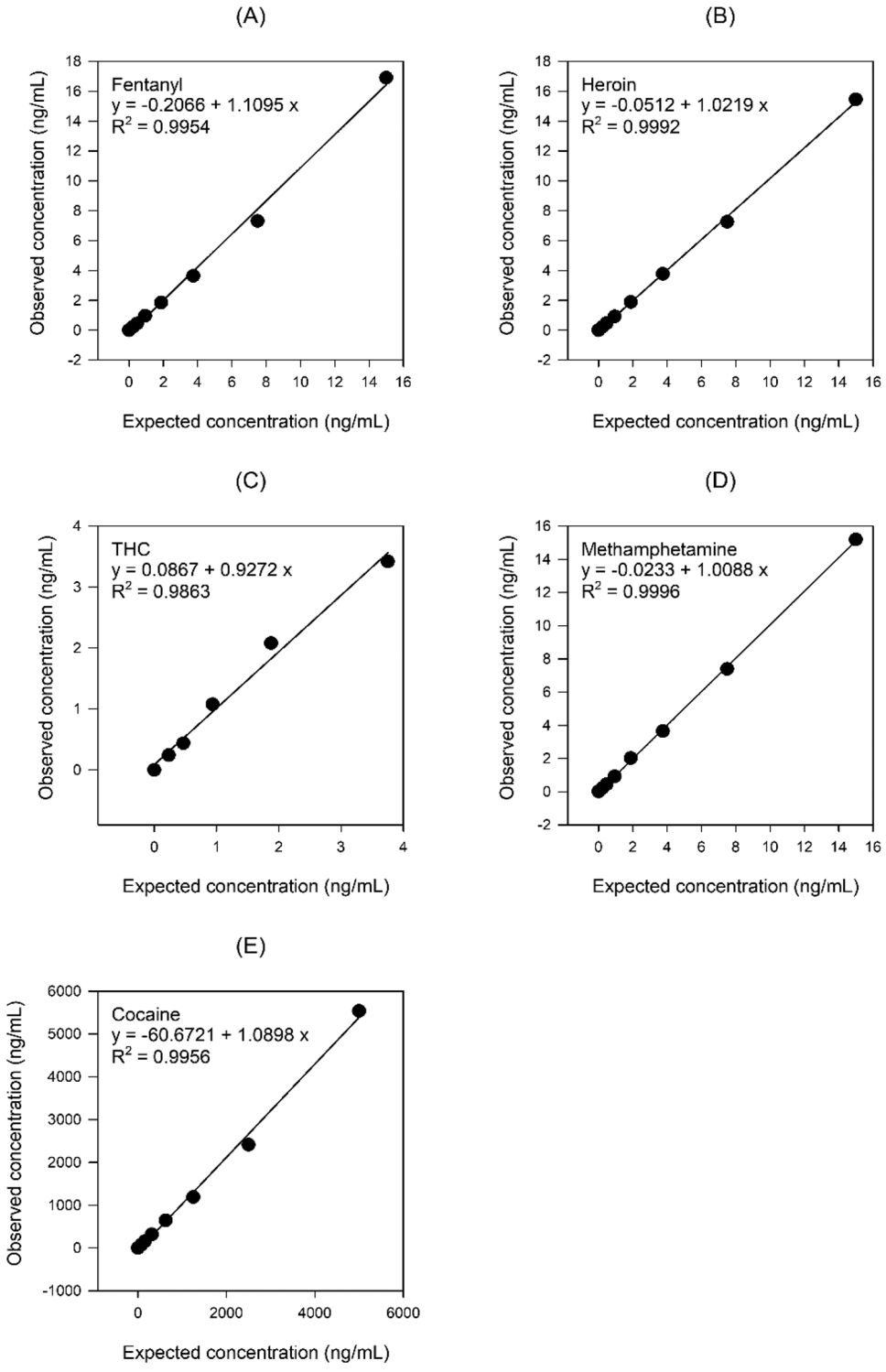

Figure 4. Observed vs expected drug concentrations. Panels A-D: Standards recovery from multidrug assay for fentanyl, heroin, THC, and methamphetamine (where observed concentrations was calculated from 4-PL fit and expected concentrations were the values of the calibration standards) Panel E: Standards recovery of multidrug assay for cocaine (where observed concentration was calculated from 4-PL fit and expected concentration was calculated from dilution of standard solution).

being performed rapidly at a relatively lower cost than other instrumental methods. This assay can be used to screen surfaces in crime laboratories, pharmacies, and drug-related law enforcement sites for contamination by drugs of abuse to improve clean-up and decontamination efforts. Routine swabs can be taken, and samples analyzed on a quarterly or annual basis as proof of proper contaminant clean-up. Crime laboratories and drug-related law enforcement sites are of particular interest because these are places where extensive drug contamination has been found in the past. In a Health Hazard Evaluation (HHE) conducted by NIOSH in 2009–2010 in a drug evidence vault, a FCMIA method, without fentanyl or its analogues, was utilized to analyze air sampling filters and surface swabs for methamphetamine, oxycodone, THC,

Table 2. Recovery percentage from spiked tiles and standard error (SE).

	Fentanyl		Heroin		THC		Methamphetamine	
ng/100 cm^2	Recovery	SE	Recovery	SE	Recovery	SE	Recovery	SE
45	26.7%	3.4%	52.5%	7.2%	OOR	OOR	44.5%	3.8%
22.5	28.5%	2.5%	44.2%	2.6%	19.6%	0.9%	63.6%	7.1%
11.25	31.8%	1.8%	40.8%	2.6%	30.5%	1.8%	65.0%	5.6%
5.63	34.0%	1.0%	43.9%	2.7%	37.6%	5.1%	88.1%	13.5%
2.81	37.2%	1.8%	37.7%	2.4%	42.4%	3.7%	101.9%	28.8%
1.41	39.8%	3.8%	40.4%	2.0%	35.3%	5.9%	72.7%	18.9%
0.7	35.0%	2.8%	33.9%	4.3%	29.2%	2.9%	124.9%	33.5%
	Cocaine							
ng/100 cm^2	Recovery	SE						
7500	53.6%	15.9%						
3750	42.6%	7.9%						
1875	39.0%	7.3%						
937.5	39.8%	6.6%						
468.8	38.6%	6.9%						
234.4	42.1%	7.9%						
117.2	37.5%	9.3%						

*OOR = Out of range of assay.

and cocaine (NIOSH et al. 2011). This and other HHEs led to recommendations for better housekeeping and safer work practices (NIOSH et al. 2011, 2013, 2021). This new method adds the ability to detect fentanyl and its analogues, so similar investigations or studies conducted during the current opioid crisis can benefit from their inclusion as analytes of interest.

One limitation of the FCMIA assay is that reagents (a protein-drug conjugate and anti-drug antibody) must be available for the drug of interest. It can be costly to develop these reagents for newer analytes, but there are several commercial laboratories that can provide conjugates and antibodies for other analytical platforms such as lateral flow immunoassay (LFIA) used to screen for illicit drug use. Once reagents are obtained, more drugs can be added to the FCMIA assay by inserting a new bead set and antibody for the drug of interest. However, commercial sources may no longer provide the desired reagents at a time in the future. The assay presented in this manuscript used an antibody that was 100 to 1000 times less sensitive for the cocaine assay than the other drugs. The original assay developed for the 2009–10 HHE used a benzoylecgonine antibody (a cocaine metabolite) that had similar sensitivity to the other drugs (Smith et al. 2010). Key reagents were no longer available and had to be replaced with less sensitive antibodies. In the future, all components could be offered in a commercially available kit that eliminates the need to purchase reagents from several vendors and with instructions written for ease of use.

There are numerous other techniques that can be applied to the measurement of surface contamination by drugs of abuse, with each having its own advantages and drawbacks. Colorimetric tests such as Marquis reactions produce rapid results, but lack specificity, with methamphetamine and fentanyl often producing similar, indistinguishable color changes (Alonzo et al. 2022). LFIAs have been used for drugs of abuse in a variety of matrices such as solid or liquid drugs, and have the advantages of portability, low cost, and near real time results (Angelini et al. 2019). When used with an assay reader, LFIA can have sensitivity equivalent to FCMIA and LC-MS/MS (Smith et al. 2015), but they usually provide measurement of one to a few drugs at a time. Instrumental methods such as LC-MS/MS and GC-MS perform a separation and an identification of analytes whereas immunochemical methods detect an analyte in a mixture. Because of this, LC and GC methods can be more sensitive and specific than immunoassays (Seymour et al. 2019). However, LC and GC instruments are more expensive to maintain and require special expertise to run. Chromatographic analyses typically require elaborate sample preparation which can increase analysis time (Seymour et al. 2019). Since immunoassays often detect classes of compounds, they are well suited for use as a screening method but may have low sensitivity which can lead to false negatives. For these reasons, chromatographic analyses may then be employed to confirm the presence of the drug.

LC and GC strengths lie in determination of individual fentanyl analogues. For instance, Ciesielski et al. (2021) describe a sampling and extraction method for 17 fentanyl analogues and 10 adulterants from surfaces. These fentanyl analogues gave a range of collection and extraction efficiencies from 34 to 82.5% and a limit of quantitation (LOQ)/lower limit of quantification (LLOQ) of 0.05 ng/100 cm^2 (Ciesielski et al. 2021). LC methods often have better surface recovery due to the ability to use organic solvents in the sampling solution that may not be compatible with immunochemical microspheres or their internal dyes (Luminex Corporation 2014). A manuscript by Horáček and Skládal (2000) shows promise that immunochemical assays can be performed when low concentrations of organic solvents such as methanol are present (2000). However, binding affinity was found to be reduced.

A GC-MS method using selected ion monitoring produced a limit of detection (LOD) of 4 ng per wipe for fentanyl and sufentanil and 16 ng per wipe for alfentanil with respective LOQs of 14, 12, and 50 ng per wipe (Van Nimmen and Veulemans 2004). The drawbacks of both LC-MS and GC-MS methods are that they are expensive to run, require specialized training to run and maintain equipment, and rely on skilled expertise for sample preparation and analysis that can

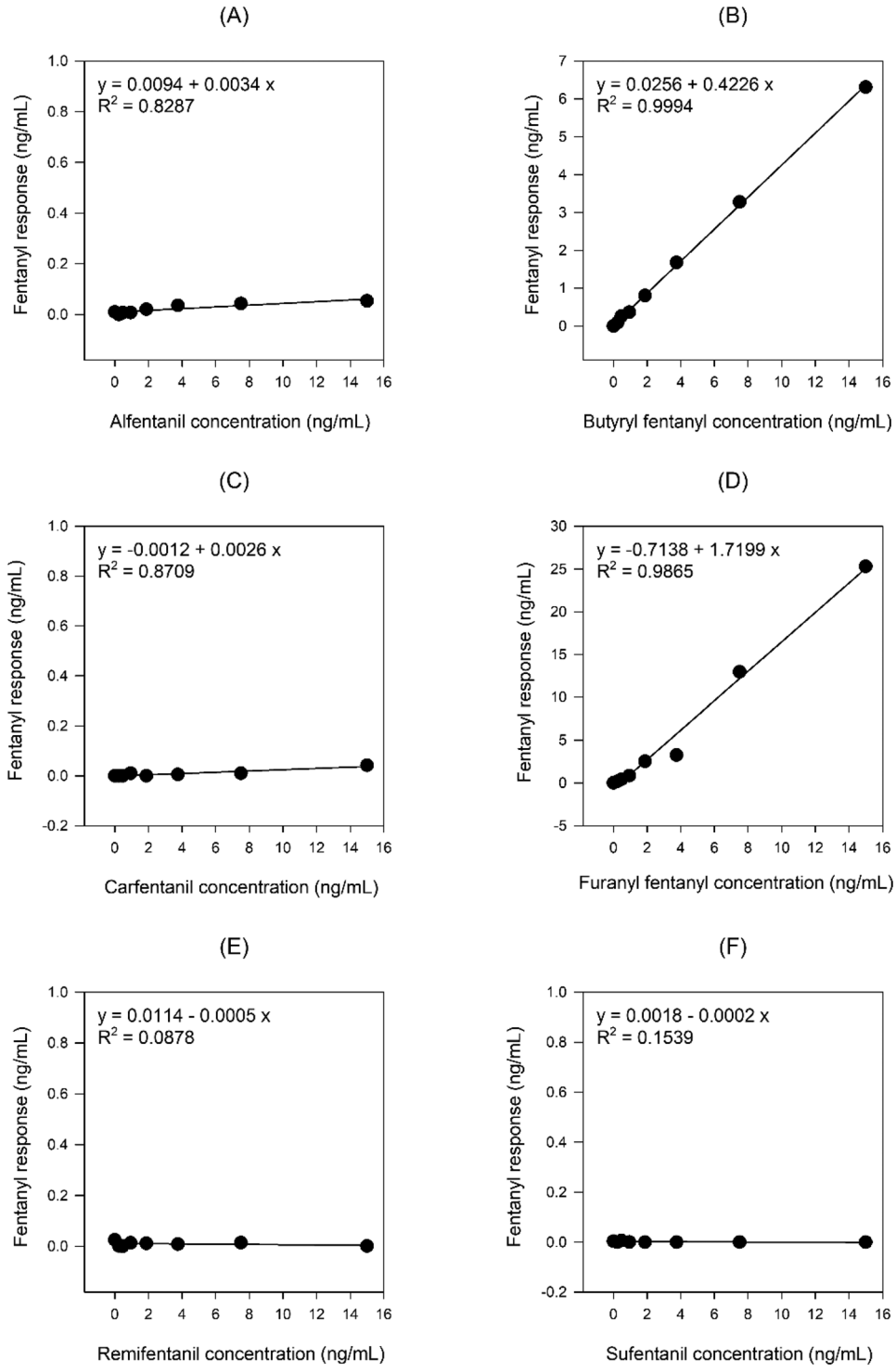

Figure 5. Response to fentanyl analogues. Panel A-F: Response of fentanyl assay to alfentanil, butyryl fentanyl (b fentanyl), carfentanil, furanyl fentanyl (fentanyl), remifentanil, sufentanil, over the range of 0–15 ng/mL for each analogue.

cause delays in obtaining results from backlogged laboratories (Valdez 2022).

Several direct reading methods such as surface-enhanced Raman spectroscopy (SERS), rapid thermal desorption direct analysis in real time mass spectrometry, (TD-DART-MS) and ion mobility spectrometry (IMS) have also been described in the literature. SERS can detect fentanyl and its analogues, but light conditions at the site can interfere with results and produce false negatives. TD-DART-MS and IMS methods can give nanogram to sub-nanogram sensitivity; however, these techniques are not considered quantitative and can produce false positives and false negatives (Sisco et al. 2019). Algorithms necessary for these techniques rely on databases of a mixture of known compounds for comparison to unknowns, and the development and evaluation process can be time-consuming (Sisco et al. 2021). These methods should be compared with more robust methods, and FCMIA provides a more cost-effective alternative to tandem LC-MS or GC-MS.

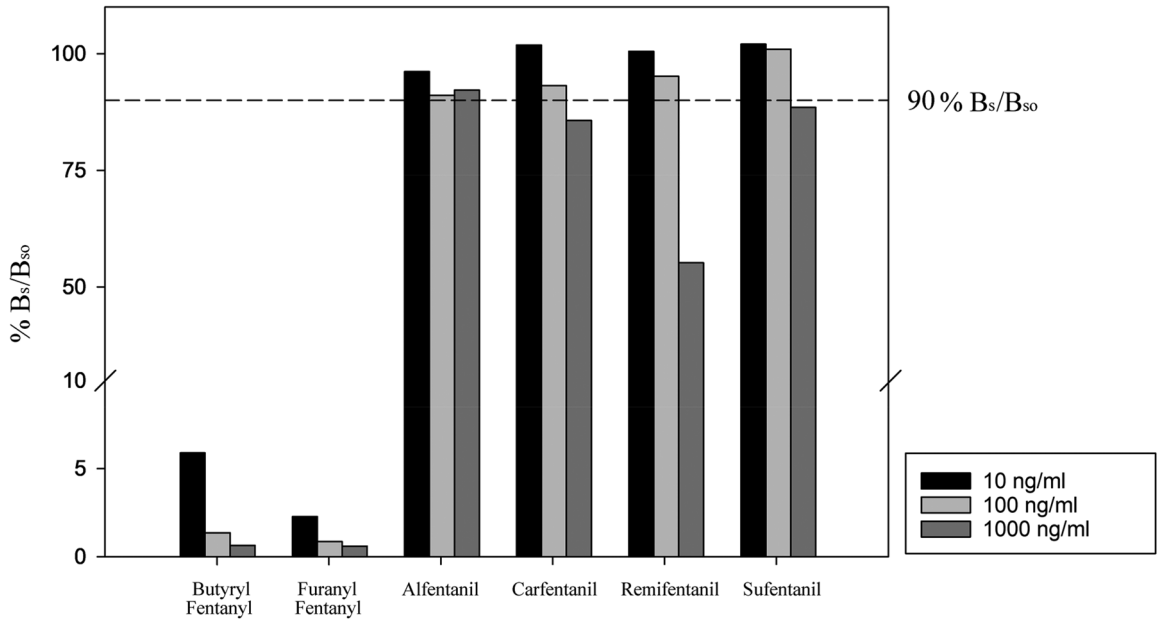

Figure 6. Fentanyl analogue drug cross-reactivity. Response of the fentanyl assay to large concentrations of fentanyl analogues (alfentanil, butyryl fentanyl, carfentanil, furanyl fentanyl, and remifentanil) between 10 and 1000 ng/mL was determined.

However, users of FCMIA must be aware of the concentration range that the test is intended, or false negatives can occur.

Fentanyl and fentanyl analogues have become an increasing hazard due to their exceptional potency compared to other drugs of abuse. Nevertheless, the other drugs of abuse still present a potential hazard to workers. A technique that can assess several drugs simultaneously is desirable. This assay is capable of detecting fentanyl and its analogues that have structural similarities to fentanyl but not to analogues that possess additional side-groups. Butyryl fentanyl and furanyl fentanyl have small changes to a side chain and their molecular shape is very similar to fentanyl. However, alfentanil, carfentanil, remifentanil and sufentanil all have a group added to the piperidine ring that significantly changes their three-dimensional structure. This deviation from the fentanyl shape may be responsible for their lower assay response. Future studies should focus on evaluating other antibodies and drug protein conjugates including aptamer-based reagents (Kammer et al. 2019) to find candidates with increased response to analogues with side-groups similar to carfentanil. The studies must also explore the use of different solvents and buffers that are compatible with the microspheres. PBS was utilized as the sampling buffer in these studies, but it may benefit from the addition of surfactants such as Tween®20 or Triton-100 to enhance recovery. Additional fentanyl analogues must be tested as well. These steps may increase the sensitivity and specificity of the assay.

Typically, immunoassay methods are designed to analyze one compound at a time, but Magpix® multiplexing enables the simultaneous analysis of up to 100 analytes. Additionally, multiplexing has the flexibility to remove or add new analytes to a method with ease as demonstrated in this paper. Multiplexing is a valuable tool for assessing exposures to an ever-changing array of dangerous drugs encountered by workers in the public safety sector.

Disclaimer

'The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of any company or product does not constitute endorsement by NIOSH'.

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Data availability statement

Following publication of the journal article, the data will be made available at https://data.cdc.gov.

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