UC San Diego
UC San Diego Previously Published Works

Title
Evaluating the performance of the Roche FEN2 fentanyl immunoassay and its clinical implementation: The role of LDT-based mass spectrometry testing

Permalink
https://escholarship.org/uc/item/1q39h1kf

Authors
Menlyadiev, Marlen
Suhandynata, Raymond T
Lund, Kyle
et al.

Publication Date
2023-04-01

DOI
10.1016/j.jmsacl.2023.02.009

Copyright Information
This work is made available under the terms of a Creative Commons Attribution-NonCommercial-NoDerivatives License, available at
https://creativecommons.org/licenses/by-nc-nd/4.0/

Peer reviewed

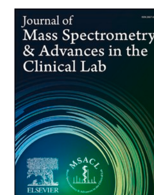
eScholarship.org Powered by the California Digital Library
University of California

Journal of Mass Spectrometry and Advances in the Clinical Lab 28 (2023) 105–113

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab



Research Article

Evaluating the performance of the Roche FEN2 fentanyl immunoassay and its clinical implementation: The role of LDT-based mass spectrometry testing

Marlen Menlyadiev [a,*], Raymond T. Suhandynata [a,b], Kyle Lund [a], Michael J. Kelner [a], Robert L. Fitzgerald [a]

[a] Department of Pathology, Center for Advanced Laboratory Medicine, University of California, San Diego Health Systems, San Diego, CA, United States
[b] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, San Diego, CA, United States

ARTICLE INFO

Keywords:
Mass spectrometry
Immunoassay
Clinical sensitivity
Fentanyl
Norfentanyl
FEN2

ABSTRACT

Introduction: While laboratory-developed tests (LDTs) using liquid chromatography tandem mass spectrometry (LC-MS/MS) are widely employed to support the development of FDA-cleared drug immunoassays, their significance in the clinical implementation and evaluation of such assays is often overlooked. This paper reports on the important role of LC-MS/MS LDTs in demonstrating improved performance of the Roche FEN2 fentanyl immunoassay compared with the Thermo DRI fentanyl immunoassay.
Methods: The FEN2 assay was implemented according to the manufacturer's instructions and its performance was compared to the existing DRI assay using LC-MS/MS as a reference. Clinical sensitivity and specificity were determined using 250 consecutive random patient specimens. Spiking experiments were conducted to determine cross-reactivity with 31 fentanyl analogs. Select DRI false-positive samples were analyzed by the FEN2 assay via time-of-flight mass spectrometry method (LC-QTOF).
Results: The FEN2 assay showed improved clinical sensitivity compared to the DRI (98% vs 61%) in 250 consecutive patient samples due to its ability to detect norfentanyl. It also showed better clinical specificity by correctly classifying select DRI false-positive results. Upon implementation in clinical practice, the FEN2 resulted in a higher screening positivity rate than the DRI (17.3% vs 13.3%) and a greater LC-MS/MS confirmation rate of immunoassay-positive samples (96.8% vs 88.8%, respectively).
Conclusion: The use of LC-MS/MS LDTs demonstrated that the FEN2 assay has greater clinical sensitivity and is less prone to false-positives than the DRI assay. These findings support the use of FEN2 in routine clinical practice and emphasize the role of mass spectrometry-based LDTs in clinical toxicology testing.

Introduction

Laboratory-developed tests (LDTs) are an integral part of modern laboratory medicine, allowing laboratorians to quickly adapt to changing patient testing needs. LDTs also facilitate the adoption of the latest technological advancements in clinical diagnostics [1]. From drug testing for emergency medicine to screening newborns for life-threatening diseases and the rapid development of SARS CoV-2 assays, LDTs play an important role in providing timely and affordable health care in the United States.

Clinical toxicology testing and urine drug screening (UDS) have a heavy reliance on LDTs [2]. The typical UDS workflow begins with rapid screening of patient samples for drug classes using automated immunoassays, followed by LDT mass spectrometry-based confirmatory testing. When dealing with challenging clinical samples, LDT liquid chromatography-high resolution mass spectrometry (LC-HRMS)

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; DFU, drug-free urine; ED, emergency department; EHR, electronic health record; EMIT, enzyme multiplied immunoassay technique; FDA, Food and Drug Administration; ICU, intensive care unit; LC-MS/MS, liquid chromatography tandem mass spectrometry; LC-QTOF, liquid chromatography-quadrupole time-of-flight mass spectrometry; LDT, laboratory-developed test; LLOQ, lower limit of quantitation; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide; NMS, National Medical Services laboratory; QC, quality control; UCSD, University of California, San Diego; UDS, urine drug screen; ULOQ, Upper limit of quantitation; UPLC, Ultra-performance liquid chromatography.

* Corresponding author.
E-mail address: mmenlyadiev@health.ucsd.edu (M. Menlyadiev).

https://doi.org/10.1016/j.jmsacl.2023.02.009
Received 1 December 2022; Received in revised form 24 February 2023; Accepted 26 February 2023
Available online 9 March 2023
2667-145X/© 2023 THE AUTHORS. Publishing services by ELSEVIER B.V. on behalf of MSACL. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

approaches can be used for broad-spectrum screening for hundreds of potential compounds [3,4].

FDA-cleared or LDT automated drug immunoassays are the mainstay of toxicology testing in clinical laboratories due to their performance, speed of analysis, and low cost. However, immunoassays have limitations, such as the inability to detect specific drugs within a class (e.g., morphine versus codeine), false-negative results due to poor analytical sensitivity and/or poor cross-reactivity with new drugs of interest within a class (e.g., buprenorphine and opiates immunoassay), false-positive results due to interferences found in patient samples, and the qualitative nature of the test. Mass spectrometry-based confirmatory tests allow laboratories to address these immunoassay limitations by selectively and quantitatively measuring drug concentrations. These LDT mass spectrometry assays do not suffer from immunoassay interferences [5] and have the flexibility to expand their test menu to accommodate the need for the detection of emerging drugs [6]. In cases with medico-legal implications (pain management clinics, pediatric patients), LDT mass spectrometry assays are the only acceptable approach to sample analysis. LC-HRMS LDT assays represent the next level of sophistication in clinical toxicology testing after targeted gas chromatography mass spectrometry (GC–MS) and LC-MS/MS methods. While targeted mass spectrometry LDTs only measure drugs they are developed to measure, LC-HRMS assays allow for a broad- spectrum or untargeted drug measurements in cases where testing for a broader range of drugs is needed (e.g., complex overdose cases with multiple or unknown drugs involved) [3,7].

Fentanyl is a potent synthetic opioid prescribed for patients with severe pain or to manage pain after surgery [8,9]. It can also be used to treat patients with chronic pain who are physically tolerant to other opioids [10]. Unfortunately, fentanyl has contributed to the opioid epidemic in North America [11,12]. Between 1999 and 2016, it was reported that>630,000 people died from drug overdoses in the US, with many of these deaths related to prescription opioids [11]. In recent years, deaths from illicitly manufactured fentanyl (IMF) have been increasing, making detection of fentanyl and related compounds a pressing issue [11].

University of California, San Diego (UCSD) Health clinical laboratories first offered fentanyl in our UDS in August 2021 as an LDT, based on the Thermo Fisher Scientific's DRI fentanyl kit [13]. When the Roche FEN2 assay [14] was cleared by the FDA in 2022, we conducted a method comparison between the two assays using LC-MS/MS as a reference method to better understand their performance characteristics. One particularly attractive feature of FEN2 was its low detection cutoff values for norfentanyl compared with other commercially available fentanyl immunoassays [13–17] (Table 1). The ability to detect low concentrations of fentanyl and norfentanyl is important due to the short elimination half-life of the parent drug and its extensive metabolism. With IV administration, for example, up to 85% of fentanyl is excreted in urine over a 3–4 day period, with only 0.4–6% eliminated as fentanyl and 26–55% as norfentanyl [18,19].

This manuscript describes the key role of LC-MS/MS LDTs in validating new immunoassays. We highlight the utility of LDT mass spectrometry-based assays as arbiters of discrepant immunoassay results and demonstrate how they can be used to improve UDS capabilities.

Table 1
Comparison of the detection cutoff values for commercially available fentanyl immunoassays.

Immunoassay	Cutoff values	
	fentanyl	nor-fentanyl
SEFRIA (IAL)	1	>1000
ARK (Ark Dx)	1	30
ARK II (Ark Dx)	1	15
DRI (Thermo)	2	10,000
DRI II (Thermo)	1	15
FEN2 (Roche)	3.8	5

Materials and methods

Specimens

To determine the clinical sensitivity and specificity, excess urine specimens from a total of 250 consecutive UDS samples (no inclusion criteria applied) were collected between May 4, 2022 and May 17, 2022 under UCSD IRB Protocol 181656. This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects. The UCSD institutional review board deemed that informed consent was not necessary because this study used existing specimens. These specimens were first screened using the DRI assay (May 4, 2022 to May 17, 2022) and then stored frozen at −20 °C until they were analyzed by the FEN2 assay (September 9, 2022 to September 10, 2022). Each specimen in the study was sent to the clinical toxicology laboratory for quantitative analysis by LC-MS/MS (November 2022) for fentanyl and norfentanyl (Fig. 1). In addition, a second set of 21 samples was collected between October 2021 and January 2022 to compare the DRI and FEN2 assays' clinical performance. These were residual urine samples that screened positive on the DRI, but were negative by LC-MS/MS (concentrations of both fentanyl and norfentanyl < 2 ng/mL). All of these DRI-false positive samples were then screened with the FEN2 assay.

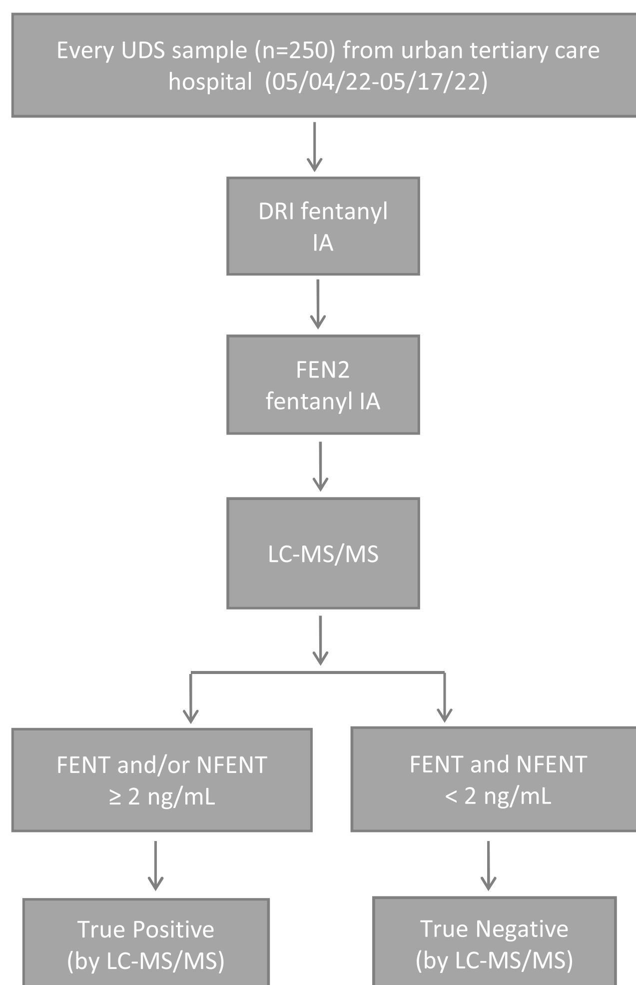


Fig. 1. Study design for clinical performance evaluation of the DRI and FEN2 assays (a positive sample was defined as true positive if it contained ≥ 2 ng/mL of fentanyl or norfentanyl and negative as true negative if the concentrations of both analytes in a sample were < 2 ng/mL).

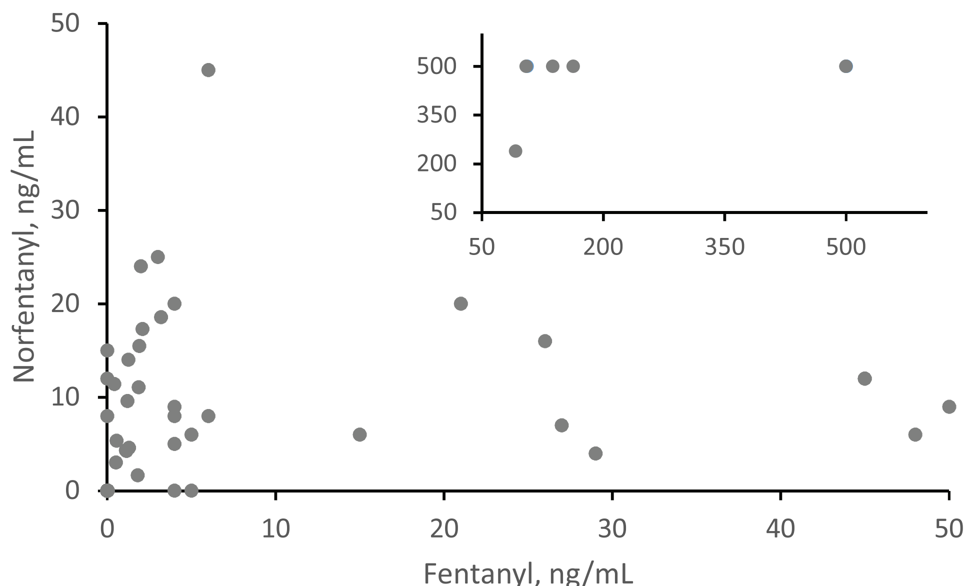


Fig. 2. Distribution of fentanyl and norfentanyl concentrations (determined by LC-MS/MS) in the study sample population (n = 250).

Table 2
Verification of the accuracy (A) and precision (B) of the FEN2 assay (Negative and positive control samples (Neg QC and Pos QC) contained 3.75 and 6.25 ng/mL of norfentanyl, respectively).

(A)

		LC-MS/MS		
		Negative reference	Positive reference	Total
FEN2	Negative test	40	0	40
	Positive test	0	40	40
	Total	40	40	80

(B)

	Within-day CV	Between-day CV	Estimated total CV
Neg QC	1.0%	1.3%	1.6%
Pas QC	0.8%	1.3%	1.5%

Cross-reactivity specimens

To evaluate the ability of the immunoassays to detect fentanyl analogs, standard solutions of 31 analogs (Cayman Chemical, Ann Arbor, MI) were individually spiked into drug-free human urine (DFU; UTAK Laboratories Inc., Santa Clarita, CA) at 1 and 10 ng/mL. Samples were tested with both immunoassays. Twenty of these analogs were the same as those found in National Medical Services (NMS) laboratory's qualitative urine screen for designer opioids (test code 1480U), excluding carfentanyl. The remaining fentanyl analogs were selected for testing based on their prevalence in seized drug samples in San Diego County (personal communication, San Diego County Sheriff's Department).

DRI and FEN2 fentanyl immunoassay

Both the DRI (Thermo Fisher Scientific) and FEN2 (Roche Diagnostics) enzyme multiplied immunoassay technique (EMIT) tests are based on competition between a drug labeled with glucose-6-phosphate dehydrogenase (G6PDH), and drug from a urine sample for a fixed amount of specific antibody binding sites. In the absence of drug from the sample, the specific antibody binds the drug labeled with G6PDH, resulting in a decrease in enzyme activity. This reaction creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is determined spectrophotometrically at 340 nm by measuring the conversion of nicotinamide adenine dinucleotide (NAD) to its reduced form NADH. The assays were implemented on the Roche Cobas c502 analyzer according to the manufacturers' instructions

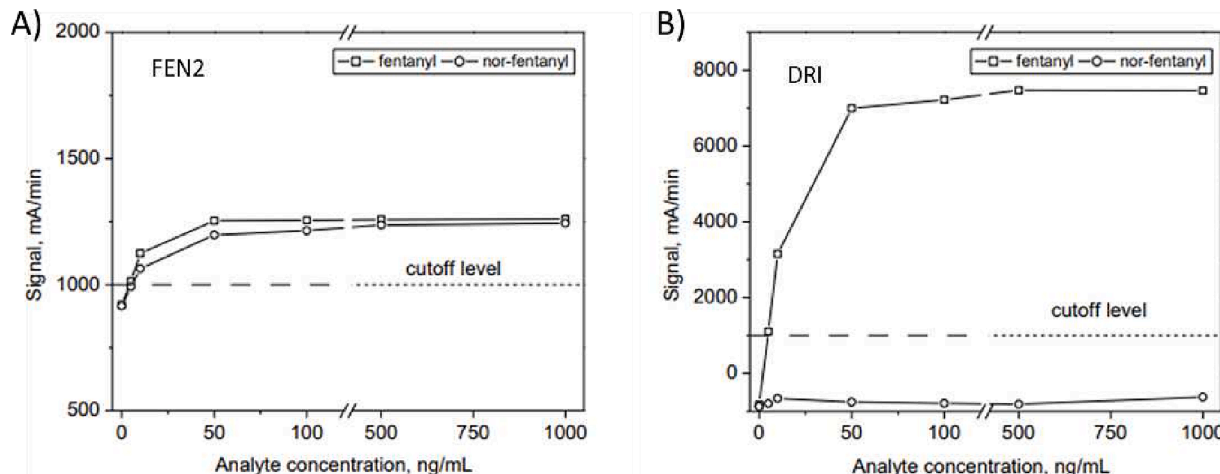


Fig. 3. Verification of the analytical measurement range (AMR) for the FEN2 (A) and the DRI (B) assays.

Table 3
Comparison of the clinical performance of the DRI (A) and the FEN2 (B) assays in 250 UDS samples sequentially collected from urban tertiary care hospital (05/04/22–05/17/22). LC-MS/MS was the reference method for calculating clinical sensitivity and specificity of the assays.

A) LC-MS/MS

DRI	-	+	Total
-	198	20	218
+	1	31	32
Total	199	51	250

B)

FEN2	-	+	Total
-	198	1	199
+	1	50	51
Total	199	51	250

C)

Assay	Sensitivity	Specificity
DRI	61%	99.5%
FEN2	98%	99.5%

[13,14]. The DRI assay was in clinical use from August 11, 2021 until August 30, 2022 when it was replaced by the FEN2 assay.

As part of the performance verification of the FEN2 assay, accuracy, precision and analytical measurement range were tested. Forty positive and forty negative patient samples were used to verify accuracy with an in-house LC-MS/MS quantitative method as the reference. Within-day and between-day precision were calculated using Roche DAT Opiates Multi Control I Set positive and negative quality control (QC) samples (containing 6.25 ng/mL and 3.75 ng/mL of norfentanyl, respectively). Five specimens were run for five days (N = 25) for both QC levels. The analytical measurement range of the FEN2 and the DRI (for comparison) assays was verified by spiking DFU with fentanyl and norfentanyl standard solutions at 5, 10, 50, 100, 500, and 1000 ng/mL. These spiked samples were tested using both assays.

Confirmatory LC-MS/MS opiates method

A confirmatory quantitative LC-MS/MS method for 14 opiates, including fentanyl and norfentanyl, was developed and validated as an in-house LDT according to CLSI guidelines [20] prior to the current work. All reagents and LC-MS grade solvents were purchased from Fisher Scientific (Waltham, MA). Deuterium-labeled internal standards (-d3 for all analytes, except fentanyl and norfentanyl which were -d5) were purchased from Cerilliant Corporation (Round Rock, TX). Fifteen microliters of urine specimens were mixed with recombinant IMCSzyme beta-glucuronidase in a hydrolysis buffer (IMCS LLC, Irmo, SC) and incubated for 30 min at 55 °C. After incubation, the sample was diluted to a final volume of 1.5 mL with deionized water, centrifuged, and injected into the LC-MS/MS. A Waters XEVO TQ-S triple quadrupole mass spectrometer with Acquity UPLC chromatograph (Waters Corporation, Milford, MA) was used for analysis. Samples were separated (Supplemental Fig. 1S) on a Waters HSS C18 2.5 um × 2.1 × 150 mm UPLC XP column with Phenomenex UPLC 2.1 mm C18 guard column (Phenomenex, Torrance, CA) using gradient elution over 4.5 min. Mobile phase A was 5 mM aqueous solution of ammonium formate at pH 3.0 and mobile phase B was 0.1% solution of formic acid in acetonitrile. The concentration of B was linearly increased from 5 to 23% in 3 min and then to 95% at 4.5 min from the start of the run. The mass

Table 4
A) DRI false-negative samples (among 250 study samples from May 4-17, 2022) and B) hospital services/wards where these samples were collected.

A)

	LC-MS/MS results, ng/mL		Immunoassay screen results	
Sample ID	*Fentanyl*	*Nor-fentanyl*	*DRI*	*FEN2*
1	<2	12	Neg	Pos
14	<2	8	Neg	Pos
34	<2	3	Neg	Pos
40	<2	11.4	Neg	Pos
107	3	55	Neg	Pos
122	<2	5.4	Neg	Pos
125	<2	15.5	Neg	Pos
136	5	6	Neg	Pos
160	2	24	Neg	Pos
164	3	25	Neg	Pos
167	6	45	Neg	Pos
170	<2	15	Neg	Pos
171	<2	4.6	Neg	Pos
172	<2	4.3	Neg	Pos
180	<2	11.1	Neg	Pos
182	<2	4.3	Neg	Pos
198	2.1	17.3	Neg	Pos
203	<2	9.6	Neg	Pos
204	3.2	18.6	Neg	Pos
230	<2	14	Neg	Pos

B)

Service/Ward	Outpatient	Postpartum care	ED	ICU	Nursery
Number	9	4	4	2	1

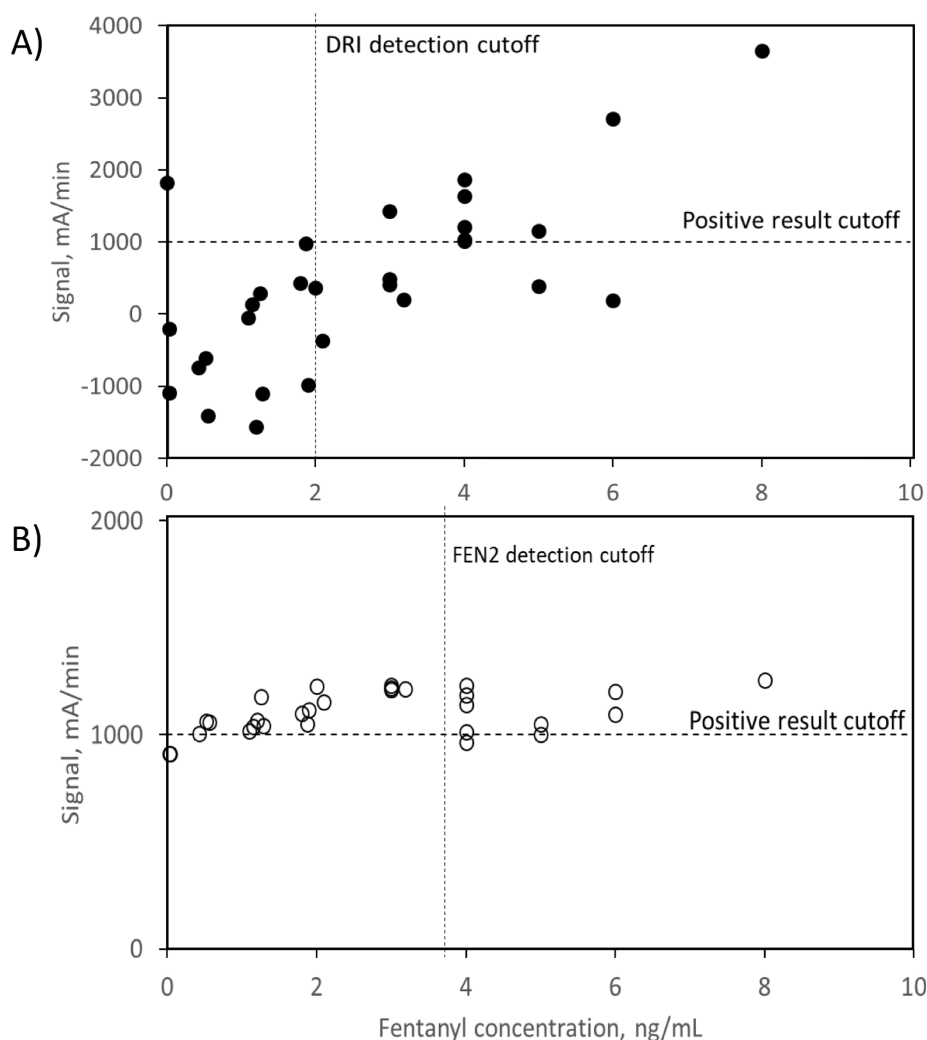


Fig. 4. Effect of inter-individual sample differences on immunoassay signal (signal ≥ 1000, positive).

spectrometer was operated in multiple reaction monitoring (MRM) mode with parameters (cone voltage and collision energy) optimized for each analyte. Analyte retention times, ion transitions, analytical measurement ranges (AMRs) and precision for opiates LC-MS/MS method are summarized in the Supplemental Table 1S. Analytes were identified based on retention times relative to internal standards and by measuring peak area ratios of quantifier and qualifier ion transitions for each analyte. Concentrations of drugs in samples were calculated using calibration curves generated by linear regression with 1/x weighting based on peak area of analyte relative to peak area of deuterium-labeled internal standard. As shown in the Supplemental Table 1S, the LLOQs for fentanyl and norfentanyl were 2.0 ng/mL; however, the LC-MS/MS opiates method was able to detect both analytes at 0.5–1 ng/mL (matching relative retention times, signal-to-noise ratio > 3, acceptable quantifier-to-qualify ion ratios, etc.).

Broad spectrum drug screening of DRI false positive specimens using LC-QTOF

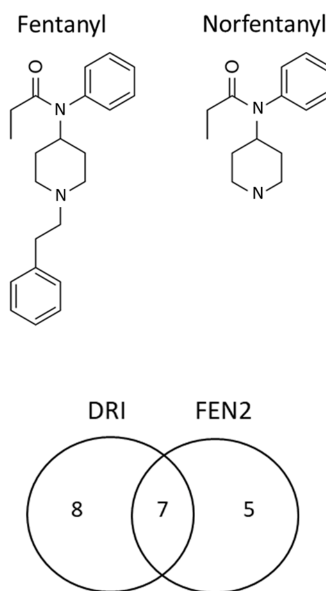
DRI false-positive samples collected between October 2021 and January 2022 were analyzed for drugs, metabolites, and related compounds, such as nutritional supplements using an in-house LC-QTOF broad-spectrum drug screening method in MS^E mode on a Xevo G2 instrument (Waters Corporation, Milford, MA). MS^E is a data-independent acquisition (DIA) approach in Waters' instruments that allows for the collection of full information on precursor and fragment ions in a single analysis by alternating between low- and high-energy fragmentation modes. This method has been described previously [3,4]. Briefly, samples were subjected to a dilute, hydrolyze, and shoot protocol. Results were processed with three sets of criteria with increasing stringency: a combination of retention time match (±0.2 min), presence of protonated analyte measured with high mass accuracy (5 ppm), and detection of at least one fragment ion with high mass accuracy (5 ppm) and sufficient intensity (>1000 counts) resulted in positive identification. Up to 10 of the most abundant analytes per sample that met these criteria are listed in the Supplemental Table 2S. The vendor-supplied library used for known-unknown identification in analyzed DRI false positive samples contained >1500 compounds.

Clinical performance evaluation of the DRI and FEN2 assays

The clinical performance of the fentanyl immunoassays was evaluated by querying UCSD Health's electronic health records (EHR) for October 2021 for the samples screened using the DRI assay and then for October 2022 for the samples screened using the FEN2 assay. During queried periods, 1075 and 1067 samples were screened by the DRI and FEN2 assays, respectively, with 143 and 185 of these samples screening positive. LC-MS/MS testing confirmed 127 and 179 of positive screens by the DRI and FEN2. Screening positivity (143/1075, 185/1067) and confirmation (127/143, 179/185) rates were calculated from these data. Boolean searches in EHR were also used to determine the number of false-positive and false-negative immunoassay screens.

Table 5
Evaluation of the DRI and the FEN2 fentanyl assays' cross-reactivity with the select list of fentanyl analogs.

Fentanyl Analog	Concentration of spiked analog in DFU			
	1 ng/mL		10 ng/mL	
	Immunoassay result			
	DRI	FEN2	DRI	FEN2
Para-Fluorofentanyl (4-Fluorofentanyl)	Neg	Neg	Pos	Neg
ß-methyl Acetyl fentanyl	Neg	Neg	Pos	Neg
Isobutyrylfentanyl	Neg	Neg	Pos	Neg
para-Fluoroisobutyrylfentanyl (FIBF)	Neg	Neg	Pos	Neg
Valeryl Fentanyl (Pentylfentanyl)	Neg	Neg	Pos	Neg
Isovaleryl fentanyl	Neg	Neg	Pos	Neg
THF-F Tetrahydrofuran fentanyl	Neg	Neg	Pos	Neg
2-Furanylfentanyl (Fu-F; Furanylfentanyl)	Neg	Neg	Pos	Neg
2'-Fluorofentanyl	Neg	Neg	Pos	Pos
3'-Fluorofentanyl	Neg	Neg	Pos	Pos
4'-Fluorofentanyl	Neg	Neg	Pos	Pos
Methoxyacetylfentanyl	Neg	Neg	Pos	Pos
Cyclopropylfentanyl	Neg	Neg	Pos	Pos
Acryl Fentanyl	Neg	Neg	Pos	Pos
Butyrylfentanyl	Neg	Neg	Pos	Pos
Para-Methylmethoxyacetylfentanyl	Neg	Neg	Neg	Pos
Meta-Methylmethoxyacetylfentanyl	Neg	Neg	Neg	Pos
para-Chlorofentanyl	Neg	Neg	Neg	Pos
meta-Fluorofentanyl	Neg	Neg	Neg	Pos
Benzyl fentanyl	Neg	Neg	Neg	Pos
3'-methyl Acetyl fentanyl	Neg	Neg	Neg	Neg
4-ANPP (despropionylfentanyl)	Neg	Neg	Neg	Neg
cis-3-Methylfentanyl	Neg	Neg	Neg	Neg
para-Fluorobutyrylfentanyl (4F-butyryl fentanyl)	Neg	Neg	Neg	Neg
ortho-Fluorofentanyl (2-Fluorofentanyl)	Neg	Neg	Neg	Neg
trans-3-Methylfentanyl	Neg	Neg	Neg	Neg
4'-methyl Acetyl fentanyl (hydrochloride)	Neg	Neg	Neg	Neg
Acetyl fentanyl (hydrochloride)	Neg	Neg	Neg	Neg
U-47700 (U-4)	Neg	Neg	Neg	Neg
U-49900	Neg	Neg	Neg	Neg
U-51754	Neg	Neg	Neg	Neg
Detection rate	0/31	0/31	15/31	12/31



Results and discussion

The key role of LDT LC-MS/MS in drug immunoassay's clinical implementation and evaluation

The opioid epidemic in the US is a significant public health issue [12]. The volume of fentanyl-laced heroin and cocaine, as well as fentanyl counterfeit pills, is likely to continue due to the ease of manufacturing and availability of precursors from Asia [21]. Clinical laboratories must be able to meet the diverse and changing testing needs of their patient populations (emergency care, pain management, and other clinical services) with high-quality results and rapid turnaround times (TAT).

Fig. 2 shows the distribution of fentanyl and norfentanyl concentrations in 250 consecutive random patient specimens submitted for UDS testing. Thirty-eight of the 250 samples were found to contain fentanyl and 49 samples-contained norfentanyl at $\geq$ 2 ng/mL concentration. Fifty-one samples contained fentanyl, norfentanyl, or both analytes at $\geq$ 2 ng/mL, with a median fentanyl and norfentanyl concentration of 5 and 15.5 ng/mL, respectively, and corresponding interquartile ranges (IQRs) of 43 and 85 ng/mL. In addition, 6 samples from the 250-sample study pool contained detectable fentanyl and/or norfentanyl (signal-to-noise ratio $>$ 3, acceptable quantifier-to-qualify ion ratios, etc.) level which were, however, below the method's LLOQ. Of these, 5 samples contained norfentanyl and 2 samples contained fentanyl. Of the 6 samples with detectable fentanyl and/or norfentanyl, 6 and 5 screened negative by the DRI and the FEN2, respectively. These findings correspond to a 22.8% prevalence of fentanyl in our study population. A previous nation-wide study [22] reported 4.0% fentanyl positivity in non-prescribed patient population (N = 295,647) and 86.0% in a fentanyl prescribed population (N = 4353). Our prevalence data thus can be explained as arising from the combination of two types of populations (prescribed and non-prescribed) in our study sample, as may be expected in an urban tertiary care hospital. With ~ 1000 UDS orders per month and ~ 200 fentanyl-positive samples expected, meeting short TATs requires using automated fentanyl immunoassays as part of the UDS workflow. For many years, such assays were not commercially available; their emergence necessitated objective evaluation of their performance against mass spectrometry based reference methods.

The FEN2 performance verification

Prior to clinical implementation, the performance of the FEN2 assay was verified. All LC-MS/MS-confirmed samples (40 positives and 40 negatives) were correctly classified (Table 2A) by the FEN2 assay. The within- and between-day precision of the assay was below 2% (Table 2B). Dose-response curves for FEN2 showed, as expected, positive classification of samples with spiked fentanyl and norfentanyl concentrations above the assay's stated cutoff points (Fig. 3A). Similarly, dose–response curves for the DRI (Fig. 3B) showed no dose-dependent response for norfentanyl as the DRI assay does not detect norfentanyl below 10,000 ng/mL [13]. The FEN2 met laboratory verification criteria for accuracy, precision, and analytical measurement range.

3. Clinical sensitivity and specificity of the DRI and FEN2 assays

An analysis of 250 study samples by the LDT LC-MS/MS assay showed a wide variation in concentrations of fentanyl and norfentanyl in the tested patient population (Fig. 2). As can be seen in the figure, there were multiple samples in the study population with undetectable fentanyl levels, but with measurable concentrations of norfentanyl. Of the 51 LC-MS/MS positive samples in the study (defined as those containing $\geq$ 2 ng/mL of fentanyl or norfentanyl), 31 and 50 were classified correctly by the DRI and FEN2, respectively (Table 3A and B).This was in contrast to the performance in classification of the 199 LC-MS/MS-confirmed true negatives samples where both immunoassays identified 198 as negative. The calculated sensitivity and specificity were 61% and 99.5% for the DRI, and 98% and 99.5% for the FEN2 assay (Table 3C). Twenty of 250 samples in the study screened as false-negative by the DRI, but were correctly classified as positive by the FEN2 assay (Table 4A). Half of these samples were from hospital services, such as postpartum care, emergency department (ED), intensive care (ICU), and nursery; the other half were from hospital outpatient clinics (Table 4B). Similarly, the FEN2 assay correctly classified 21 DRI false-positive samples (collected from October 2021 to January 2022) as negative.

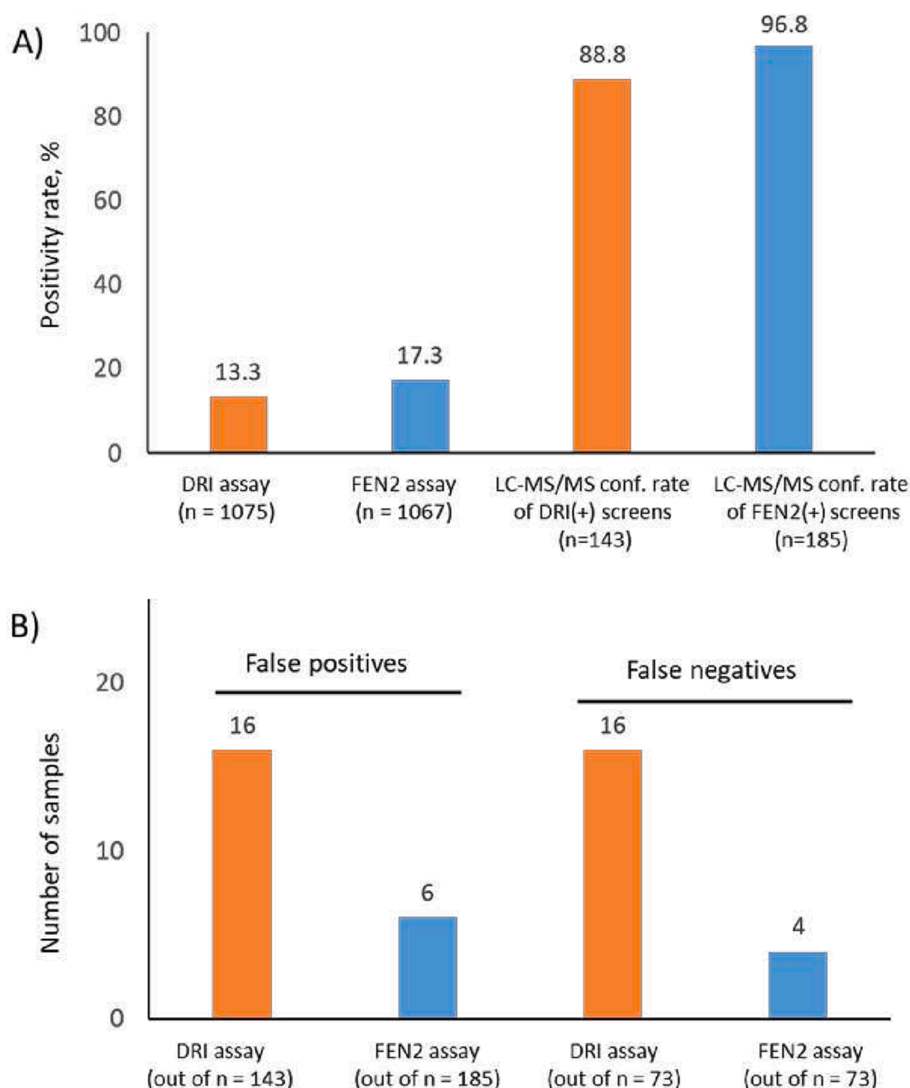


Fig. 5. Clinical performance of the DRI and FEN2 assays (data are shown for October 2021 for the DRI and October 2022 for the FEN2). Immunoassay screening and LC-MS/MS confirmation positivity rates (A) and numbers of false positives and false negatives (B) for two assays are presented.

ED, ICU, and outpatient clinics accounted for 85% of these DRI false-positive samples, with the remaining 15% coming from geriatric care, oncology, and psychiatry. One sample from the 250-sample study pool was estimated to contain fentanyl and norfentanyl at concentrations of 1.8 and 1.7 ng/mL, respectively. While technically true negative per our definition, this fentanyl- and norfentanyl-containing sample was classified as positive by the FEN2 due to its ability to detect both analytes with similar cross-reactivity [14]. This sample represents the one false positive result for the FEN2 in Table 3B. Our LC-MS/MS method is capable of detecting fentanyl and norfentanyl at 0.5–1 ng/mL concentrations (LOD); however, our clinical EHR-reportable cutoff level has been set at 2 ng/mL for years. We thus chose to continue using this 2 ng/mL LC-MS/MS cutoff for consistency when comparing data in this study with data routinely reported in the UCSD Health EHR. This could lead to a small percentage of missed fentanyl- and/or norfentanyl-positive samples during EHR queries and constitutes a possible limitation of the study.

Inter-individual sample differences in the study population were evaluated as a source of erroneous immunoassay screening results, as shown in Fig. 4. The analyzer signal (in mA/min) was plotted against LC-MS/MS-determined fentanyl concentration in a sample. As can be seen from the figure, the same fentanyl concentrations in samples from different patients yielded different analyzer signal variations for the DRI and FEN2 assays. This resulted in false positive (for DRI) and false negative (mostly for DRI but also one for FEN2) screening results. While all but one sample with fentanyl concentrations above the assay's cutoff value screened positive by FEN2 (Fig. 4B), six samples above the DRI's cutoff screened falsely negative (Fig. 4A). Positive screens for samples below FEN2's cutoff levels can be explained by the presence of norfentanyl in these samples, which is detectable by the FEN2, but not the DRI assay.

Broad spectrum drug screening of DRI false positive specimens using LC-QTOF

All immunoassays, whether FDA-cleared or not, suffer from interferences [5,23], which may have implications for patient care. We collected 21 samples that screened positive for fentanyl by the DRI assay, but did not confirm with LC-MS/MS testing. These samples were analyzed with another LDT, the LC-QTOF broad-spectrum drug screening assay. The results of the LC-QTOF testing are shown in the Supplemental Table 2S Up to 10 identified known-unknowns per sample were included in the table in order of decreasing analyte signal/abundance. The LC-QTOF method used for screening generally had limits of detection of 5–100 ng/mL, depending on the analyte and the complexity of the urine matrix. Several observations can be made from Table 2S.

First, all samples contained significant numbers of drugs, drug metabolites, or endogenous molecules, such as tryptophan. Second, risperidone and its hydroxylated metabolite were detected in four out of 21 samples; these analytes were previously reported to cause false positive screens by the DRI assay [24].

Detection of fentanyl analogs by the immunoassays

Both similarities and differences were noted in the performance evaluation of the DRI and FEN2 assays for detecting fentanyl analogs (Table 5): 7 of the 31 tested analogs (2′-fluorofentanyl, 3′-fluorofentanyl, 4′-fluorofentanyl, methoxyacetylfentanyl, cyclopropylfentanyl, butyrylfentanyl, and acryl fentanyl) were detected (positive fentanyl screen) by both assays at 10 ng/mL in DFU; 8 analogs (*para*-fluorofentanyl, ß-methyl acetyl fentanyl, isobutyrylfentanyl, *para*-fluoroisobutyrylfentanyl, valeryl fentanyl, isovaleryl fentanyl, tetrahydrofuran fentanyl and 2-furanylfentanyl) were detected only by the DRI assay; and 5 analogs (*para*-methylmethoxyacetylfentanyl, *meta*-methylmethoxyacetylfentanyl, *para*-chlorofentanyl, *meta*-fluorofentanyl and benzyl fentanyl) were detected only by the FEN2 assay. Neither assay was able to detect 3′-methyl acetyl fentanyl, 4-ANPP (despropionylfentanyl), *cis*-3-methylfentanyl, *para*-fluorobutyrylfentanyl, *ortho*-fluorofentanyl, *trans*-3-methylfentanyl, 4′-methyl acetyl fentanyl, acetyl fentanyl, U-47700, U-49900 or U-51754 at 10 ng/mL spiked concentration. None of the analog-spiked samples screened positive at 1 ng/mL concentration. With new fentanyl analogs emerging and many immunoassays being unable to detect many of them, expansion of LDT LC-MS/MS confirmatory menus or use of broad spectrum LC-HRMS drug screening will likely be important in clinical laboratories.

Clinical performance evaluation

Fig. 5 was generated by querying the EHR. Approximately the same number of total fentanyl screens were performed one month after the clinical launch of each assay: 1075 by the DRI (October 2021) and 1067 by the FEN2 (October 2022). The overall positivity rates with the DRI and FEN2 assays during this period were 13.3% and 17.3%, respectively, with corresponding LC-MS/MS confirmation rates for immunoassay-positive samples of 88.8% and 96.8%, respectively (Fig. 5A). Higher immunoassay positivity rate for FEN2 was likely due to its ability to detect norfentanyl, as was shown in the study samples (Tables 3 and 4). The false positive rates for DRI and FEN2 in these cohorts were 11.2% and 3.2%, respectively. Higher false positive rates for the DRI assay are probably due to its greater susceptibility to inter-individual differences in patient samples (Fig. 4) and drug interferences [24]. Estimated false-negativity rates (using a smaller subset of total immunoassay screens of 73 samples that were negative on a fentanyl screen, but were reflexed to LC-MS/MS analysis due to positivity on traditional opiate immunoassay screen) were 22% and 5.5% for DRI and FEN2, respectively (Fig. 5B).

Conclusions

LDT LC-MS/MS and LC-QTOF methods employed in this work allowed for an objective evaluation of the novel FEN2 fentanyl immunoassay and its comparison to the previously used DRI assay. The FEN2 met the laboratory's performance criteria and showed clear improvement in clinical performance as compared to the DRI assay, correctly classifying specimens that were false positive and false negative by the DRI. Understanding the performance characteristics of the fentanyl immunoassays in this work would not have been possible without the use of LDT-based mass spectrometry techniques, demonstrating their key role in laboratory medicine.

IRB and ethics statement

The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects. The UCSD IRB (protocol 181656) deemed that informed consent was not necessary because this study used existing specimens.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank all the staff in the UC San Diego Health clinical laboratories for their help identifying specimens for clinical sensitivity and specificity testing. We also thank Amy Rockefeller, Mabel Salarda, Ernestine Ferrer, Dawn Francisco, Nicholas Asfour, Heather Tone, and Joseph Anderson for valuable technical expertise and help with the project. We would like to thank Waters Corporation and Roche Diagnostics for supporting the clinical chemistry fellowship at the University of California San Diego.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.02.009.

References

[1] J.R. Genzen, Regulation of laboratory-developed tests, Am. J. Clin. Pathol. 152 (2) (2019) 122–131, https://doi.org/10.1093/ajcp/aqz096.
[2] P.J. Jannetto, R.L. Fitzgerald, Effective use of mass spectrometry in the clinical laboratory, Clin. Chem. 62 (1) (2016) 92–98, https://doi.org/10.1373/clinchem.2015.248146.
[3] N.S. Chindarkar, M.R. Wakefield, J.A. Stone, R.L. Fitzgerald, Liquid chromatography high-resolution TOF analysis: investigation of MS^E for broad-spectrum drug screening, Clin. Chem. 60 (8) (2014) 1115–1125, https://doi.org/10.1373/clinchem.2014.222976.
[4] Metushi IG, Wakefield MR, Lynch K, Stone J, Fitzgerald RL. Reproducibility for a Broad Spectrum Drug Screening Method from Urine using Liquid Chromatography Time-of-Flight Mass Spectrometry. Clin Mass Spec 2018; 8:1-7.19.
[5] A. Saitman, H.D. Park, R.L. Fitzgerald, False-positive interferences of common urine drug screen immunoassays: a review, J. Anal. Toxicol. 38 (7) (2014) 387–396.
[6] C.W. O'Connell, C.A. Sadler, V.M. Tolia, B.T. Ly, A.M. Saitman, R.L. Fitzgerald, Overdose of etizolam: the abuse and rise of a benzodiazepine analog, Ann. Emerg. Med. 65 (4) (2015) 465–466.
[7] K. Lund, P. Srihari, R.T. Suhandynata, K. Schwartz, R.L. Fitzgerald, Kreshak AA qua-alluding to the past: a case of methaqualone analog ingestion, J. Anal. Toxicol. 21 (2021) bkab103.
[8] D.A. Volpe, G.A.M. Tobin, R.D. Mellon, et al., Uniform assessment and ranking of opioid Mu receptor binding constants for selected opioid drugs, Regul. Toxicol. Pharm. 59 (3) (2011) 385–390, https://doi.org/10.1016/j.yrtph.2010.12.007.
[9] L. Nelson, R. Schwaner, Transdermal fentanyl: pharmacology and toxicology, J. Med. Toxicol. 5 (4) (2009) 230–241, https://doi.org/10.1007/BF03178274.
[10] K.P. Garnock-Jones, Fentanyl buccal soluble film: a review in breakthrough cancer pain, Clin. Drug Invest. 36 (5) (2016) 413–419, https://doi.org/10.1007/s40261-016-0394-y.
[11] Bernard SA, Chelminski PR, Ives TJ, Ranapurwala SI. Management of Pain in the United States-A Brief History and Implications for the Opioid Epidemic. Health Serv Insights. 2018;11:1178632918819440. Published 2018 Dec 26. 10.1177/1178632918819440.
[12] S.R. Delaney, D.H. Tacker, C.L.H. Snozek, The North American opioid epidemic: opportunities and challenges for clinical laboratories, Crit. Rev. Clin. Lab. Sci. 59 (5) (2022) 309–331, https://doi.org/10.1080/10408363.2022.2037122.
[13] https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FCDD%2FPackage-Inserts%2F10016007-DRI-Fentanyl-Assay-CJF-EN.pdf.
[14] FEN2 (Fentanyl II Enzyme Immunoassay) Package Insert, V1.0/2021-11, Roche Diagnostics, 2021.
[15] M.S. Tang, M. Lloyd, M. Williams, C.W. Farnsworth, M.M. Budelier, Performance evaluation of an automated fentanyl immunoassay, J. Appl. Lab. Med. 6 (5) (2021) 1192–1201, https://doi.org/10.1093/jalm/jfab033.
[16] S. Feng, T.J. Rutledge, M. Manzoni, et al., Performance of two fentanyl immunoassays against a liquid chromatography-tandem mass spectrometry method, J. Anal. Toxicol. 45 (2) (2021) 117–123, https://doi.org/10.1093/jat/bkaa053.

[17] M.M. Budelier, C.E. Franks, N. Logsdon, et al., Comparison of two commercially available fentanyl screening immunoassays for clinical use, J. Appl. Lab. Med. 5 (6) (2020) 1277–1286, https://doi.org/10.1093/jalm/jfaa048.

[18] D.A. McClain, C.C. Hug Jr., Intravenous fentanyl kinetics, Clin. Pharmacol. Ther. 28 (1) (1980) 106–114.

[19] Baselt RC. Disposition of Toxic Drugs and Chemicals in Man. 10th ed. Biomedical Publications: Seal Beach, California 2014; 846-849.

[20] CLSI, Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline, Clinical and Laboratory Standards Institute, 2014. CLSI document C62-A.

[21] P. Armenian, K.T. Vo, J. Barr-Walker, K.L. Lynch, Fentanyl, fentanyl analogs and novel synthetic opioids: a comprehensive review, Neuropharmacology 134 (Pt A) (2018) 121–132.

[22] J.D. Stanton, P. Whitley, L. LaRue, W.L. Bundy, E. Dawson, A. Huskey, Fentanyl analog positivity among near-real-time urine drug test results in patients seeking health care, Drug Alcohol Depend. 217 (2020), 108264.

[23] K.A. Geno, A. Badea, K.L. Lynch, et al., An opioid hiding in plain sight: loperamide-induced false-positive fentanyl and buprenorphine immunoassay results, J. Appl. Lab. Med. 7 (6) (2022) 1318–1328.

[24] B.T. Wang, J.M. Colby, A.H. Wu, K.L. Lynch, Cross-reactivity of acetylfentanyl and risperidone with a fentanyl immunoassay, J. Anal. Toxicol. 38 (9) (2014) 672–675, https://doi.org/10.1093/jat/bku103.