Title

Comparison of two highly sensitive benzodiazepine immunoassay lab developed tests for urine drug testing in clinical specimens

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Clinical implementation and performance evaluation of novel Roche FEN2 fentanyl immunoassay: the key role of LDT-based mass spectrometry testing

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Abstract

Introduction

While LC-MS/MS laboratory-developed tests (LDTs) are widely used to support the development of FDA-cleared drug immunoassays, their significance in the clinical implementation and evaluation of such assays is less recognized and appreciated. In this work we report on the key role of LC-MS/MS LDT in demonstrating improved performance of the Roche FEN2 fentanyl immunoassay as compared with the DRI fentanyl immunoassay.

Methods

The FEN2 assay was implemented per manufacturer's instructions, and its performance compared to existing DRI assay using LC-MS/MS as a reference. Clinical sensitivity and specificity were determined using 250 consecutive random patient specimens. Cross-reactivity with 31 fentanyl analogs was determined in spiking experiments. Select DRI-false-positive (FP) samples were analyzed by FEN2 assay and LC-QTOF method.

Results

The FEN2 assay showed improved clinical sensitivity over that of the DRI (98% vs 61%) in 250 consecutive patient samples, primarily due to its ability to detect norfentanyl. It also showed better performance at correctly classifying select DRI false positive results. Implementation of the FEN2 in clinical practice resulted in both higher screening positivity rate compared to the DRI (17.3% vs 13.3%) and greater LC-MS/MS confirmation rate of immunoassay-positive samples (96.8% vs 88.8%, respectively).

Conclusion

The use of LC-MS/MS LDT enabled us to demonstrate that the FEN2 assay has greater clinical sensitivity and is less prone to false positive results as compared with the DRI assay. These findings support the implementation of the FEN2 in a routine clinical practice and underline the important role of mass spectrometry-based LDTs in clinical toxicology testing.

List of Abbreviations Used: 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; FN, false negative; FP, false positive; 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.

Keywords: mass spectrometry, immunoassay, clinical sensitivity, fentanyl, norfentanyl, FEN2

1. Introduction

Laboratory-developed tests (LDTs) are an integral part of modern laboratory medicine that allows laboratorians to rapidly adapt to the changing testing needs of their patient populations. LDTs enable the adoption of the latest technical advancements in the field of clinical diagnostics [1]. From drug testing in support of emergency medicine, to screening newborns for life-threatening diseases, and the rapid development of SARS CoV-2 assays, LDTs play an important role in delivering timely and affordable health care in the United States.

Clinical toxicology testing and urine drug screening (UDS) are among the areas of laboratory medicine with the heavy reliance on LDTs [2]. In the typical UDS general workflow, rapid screening of patient samples for drug classes using automated immunoassays is generally followed by LDT mass spectrometry based confirmatory testing. With more challenging clinical samples, LDT liquid chromatography-high resolution mass spectrometry (LC-HRMS) approaches can be used for broad spectrum screening for hundreds of potential compounds .

FDA-cleared or LDT automated drug immunoassays are the mainstay of toxicology testing by clinical laboratories because of their performance, speed of analysis, and low cost. Immunoassays have limitations such as inability to detect specific drugs in a class (e.g. morphine vs codeine), poor cross-reactivity with a new drugs of interest within a class (e.g. buprenorphine and opiates immunoassay), false-positive (FP) 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 a quantitatively measuring drug concentrations. Mass spectrometry based LDTs do not suffer from immunoassay interferences and have flexibility for test menu

expansion to accommodate the need for the detection of emerging drugs. 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 GC-MS and LC-MS/MS. In cases where testing for a broad range of drugs is needed (e.g. complex overdose cases with multiple or unknown drugs involved), LC-HRMS testing allows clinical laboratories to detect much broader spectrum of drugs in patient samples than GC-MS or LC-MS/MS approaches.

Fentanyl is a potent synthetic opioid prescribed for patients with severe pain or to manage pain after surgery [3, 4]. It can also be used to treat patients with chronic pain who are physically tolerant to other opioids [5]. Unfortunately, fentanyl also contributes to the opioid epidemic in North America [6, 7]. It was reported that between 1999 and 2016, more than 630,000 people died from drug overdoses in the US, with most of these deaths related to prescription opioids [6]. In more recent years deaths from illicitly manufactured fentanyl (IMF) have been on the rise, making detection of fentanyl and related compounds a pressing issue [6].

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 [8, 9]. When the Roche FEN2 assay [10] was cleared by the FDA in 2022, we did a method comparison between the two assays using LC-MS/MS as a reference method to better understand the immunoassays performance characteristics. One particularly attractive feature of FEN2 was the low detection cutoff values for norfentanyl as compared with other commercially available fentanyl immunoassays [8, 10, 11, 12, 13] **(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 3-4 day period with only 0.4-6 % eliminated as fentanyl and 26-55% as nor-fentanyl [14, 15].

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

2. Materials and Methods

2.1. Specimens

To determine the clinical sensitivity and specificity, excess urine specimens from a total of 250 consecutive UDS were collected between 05/04/22 and 05/17/22 under UCSD IRB protocol 181656. 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 institutional review board deemed that informed consent was not necessary because this study used existing specimens. These 250 consecutive random patient specimens (no inclusion criteria applied) were first screened using the DRI assay (05/04/22- 05/17/22) and then stored frozen at -20°C until they were analyzed by the FEN2 assay (09/09/22-09/10/22). Each specimen in the study was sent to the clinical toxicology laboratory for quantitative analysis by LC-MS/MS (11/22) for fentanyl and norfentanyl **(Figure 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 of fentanyl and norfentanyl < 2 ng/mL). All of these DRI-false positive samples were then screened by the FEN2 assay.

2.2. Cross-reactivity specimens

To evaluate the ability of the immunoassays to detect fentanyl analogs, standard solutions of thirty-one 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 and samples were tested with both immunoassays. Twenty of these analogs were the same as 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).

2.3. DRI and FEN2 fentanyl immunoassay

Both DRI (Thermo Fisher Scientific) and FEN2 (Roche Diagnostics) are enzyme multiplied immunoassay technique (EMIT) tests that 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 and causes 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 NADH. The assays were implemented on Roche Cobas c502 analyzer according to manufacturers' instructions [8, 10]. The DRI assay was in clinical use from 08/11/2021 until 08/30/2022 when it was replaced with the FEN2 assay.

As part of FEN2 assay's performance verification, method accuracy, precision and analytical measurement range were tested. Accuracy was verified using 40 positive and 40 negative patient samples with 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) by running 5 specimens for 5 days (N=25) for both QC levels. 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. Spiked samples were tested using both assays.

2.4. Confirmatory LC-MS/MS opiates method

Confirmatory quantitative LC-MS/MS method for 14 opiates (including fentanyl and norfentanyl) had been developed and validated in-house as LDT using CLSI guidelines [16] prior to the current work. All reagents and LC-MS grade solvents were purchased from Fisher Scientific (Waltham, MA). Method included addition of deuterium labeled internal standards (-d3 for all analytes, but fentanyl and norfentanyl which were -d5) purchased from Cerilliant Corporation (Round Rock, TX) to 15 µL of urine specimens. Sample were then mixed with recombinant IMCSzyme beta-glucuronidase in the hydrolysis buffer (IMCS LLC, Irmo, SC) and incubated for 30 minutes at 55°C. After incubation, sample were diluted to the final volume of 1.5 mL with deionized water, centrifuged, and injected into the LC-MS/MS. Waters XEVO TQ-S triple quadrupole mass spectrometer with Acquity UPLC chromatograph (Waters Corporation, Milford, MA) was used for analysis. Samples were separated **(Supplemental Figure 1S)** on Waters HSS C18 2.5 um x 2.1 x 150 mm UPLC XP column with Phenomenex UPLC 2.1 mm C18 guard column (Phenomenex, Torrance,

CA) in 4.5 minutes using gradient elution. Mobile phase A was 5mM aqueous solution of ammonium formate at pH 3.0 and mobile phase B was 0.1% solution of formic acid in acetonitrile. During chromatographic run, the concentration of B was linearly increased from 5 to 23% in 3 minutes, and then to 95% at 4.5 minutes from the start of the run. The mass 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.

2.5. Broad spectrum drug screening of DRI False Positive specimens using LC-QTOF

DRI false positive (FP) samples collected between October 2021 and January 2022 were analyzed for drugs, metabolites and related compounds such as nutritional supplements using in-house LC-QTOF broad spectrum drug screening method in MS^E mode on Xevo G2 instrument (Waters Corporation, Milford, MA). MS^E is data-independent acquisition (DIA) approach in Waters' instruments that allows collection of full information on precursor and fragment ions in single analysis by alternating between low- and high-energy fragmentation modes. This method was describedpreviously [17, 18]. Briefly, samples were subjected to a dilute, hydrolyze, and shoot protocol. Results were processed with 3 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 most abundant analytes per samples that met these criteria are listed in the **Supplemental Table 2S**. The vendor-supplied library used for known-unknown identification in analyzed DRI-FP samples contained more than 1500 compounds.

2.6. Clinical performance evaluation of the DRI and FEN2 assays

The clinical performance of the fentanyl immunoassays was evaluated by querying UCSD Health electronic health records (EHR). The EHR was queried for numbers of samples screened using the DRI assay and then for the FEN2 assay.

3. Results and Discussion

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

The opioid epidemic in the US continues to be a significant public health issue [7]. The increase in the volumes of IMF-laced heroin and cocaine and fentanyl counterfeit pills is likely to continue for some time due to the ease of manufacturing and availability of precursors from Asia [19], requiring adequate laboratory testing strategies. Clinical laboratories should be able to meet the diverse and changing testing needs of their patient populations (emergency care, pain management and other clinical services) by providing high quality results with quick turnaround times (TAT).

Figure 2 shows the distribution of fentanyl and norfentanyl concentrations in 250 consecutive random patient specimens submitted for UDS testing. Thirty-eight of

250 samples were found to contain fentanyl and 49 samples - norfentanyl at ≥ 2ng/mL concentration. Fifty-one samples contained fentanyl, norfentanyl or both analytes at ≥ 2ng/mL. The median fentanyl and norfentanyl concentrations in these 51 samples were 5 and 15.5 ng/mL, respectively, with corresponding inter-quartile ranges (IQRs) of 43 and 85 ng/mL. In 6 study samples fentanyl and/or norfentanyl were detected at estimated concentrations below 2 ng/mL (0.6-1.8 ng/mL). Of these, 5 samples contained norfentanyl at 1.0-1.8 ng/mL and 2 samples - fentanyl at 0.6 and 1.8 ng/mL concentration. These findings correspond to 20.4-22.8% prevalence of fentanyl in our study population. A previous nation-wide study [20] reported 4.0% fentanyl positivity in non-prescribed patient population (N=295,647) and 86.0% in fentanyl prescribed population (N=4353). Our prevalence results can likely 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 the 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 and their emergence necessitated objective evaluation of their performance against mass spectrometry based reference methods.

3. 2. FEN2 performance verification

Prior to clinical implementation, the performance of the FEN2 assay was verified. All LC-MS/MS-positive samples (40 positives and 40 negative residual patient samples) 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 (Figure 3A). Such

curves were also generated for the DRI (Figure 3B) and showed no dose-dependent response for norfentanyl as the DRI assay does detect norfentanyl below 10,000 ng/mL [8]. The FEN2 met laboratory's verification criteria for accuracy, precision and analytical measurement range.

3. 3. Clinical Sensitivity and Specificity of the DRI and FEN2 assays

As noted previously, the analysis of 250 study samples by the LDT LC-MS/MS assay showed the wide variation of concentrations of fentanyl and norfentanyl in the tested patient population **(Figure 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 that contained ≥2 ng/mL of fentanyl or norfentanyl), 31 and 50 were classified correctly by the DRI and the FEN2, respectively **(Table 3, A 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 falsely negative (FN) 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 remaining half were from hospital's outpatient clinics **(Table 4B)**. Similarly, the FEN2 assay correctly classified 21 DRI false-positive samples (collected from October 2021 till January 2022) as negative**.** ED, ICU and outpatient clinics accounted for 85% of these DRI false-positive samples with 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 the assay's ability to detect both fentanyl and norfentanyl with similar cross-reactivity for both analytes [10]. This sample represents the one FP result for the FEN2 in the Table 3B. While our LC-MS/MS method is capable of detecting fentanyl and norfentanyl at 0.5-1 ng/mL concentrations (LOD), our clinical EHR-reportable cutoff level has been set at 2 ng/mL for number of years. We chose to continue using the 2 ng/mL LC-MS/MS cutoff for consistency in comparing data in this study and data routinely reported in UCSD Health EHR. This can, strictly speaking, lead to small percentage of missed fentanyl- and/or norfentanyl-positive samples during EHR query and constitutes one 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 the **Figure 4**. For this, 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 falsely positive (for DRI) and falsely negative (mostly for DRI but also one for FEN2) screening results. While all but one samples with fentanyl concentrations above assay's cutoff value screened positive by FEN2 **(Figure 4B)**, six samples above the DRI's cutoff screened falsely-negative **(Figure 4C)**. 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.

3. 4. Broad spectrum drug screening of DRI-FP specimen using LC-QTOF

All immunoassays, whether FDA-cleared or not, suffer from interferences [21] and this 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 by another LDT, LC-QTOF broad-spectrum drug screening assay to ensure that they did not have fentanyl analogs not detected by the targeted LC-MS/MS assay. The results of the LC-QTOF testing are shown in the **Supplemental Table 2S**. Up to 10 identified known-unknowns per samples were included in the table in the order of decreasing analyte signal/abundance. The LC-QTOF method used for screening [17, 18] 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 the **Table 2S**. First, all samples contained significant number of drugs, drug metabolites or endogenous molecules such as tryptophan. Noteworthy here is that in 4 out of 21 samples, risperidone and its hydroxylated metabolite were detected. These analytes were previously reported to cause FP screens by the DRI assay [8, 9].

3. 5. Detection of Fentanyl Analogs by the Immunoassays

Both similarities and differences in the detection of fentanyl analogs by DRI and FEN2 were noted in the process of the assays performance evaluation **(Table 5)**: 7 of 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) – by the DRI assay only, and 5 analogs (para-methylmethoxyacetylfentanyl, meta-methylmethoxyacetylfentanyl, para-

chlorofentanyl, meta-fluorofentanyl and benzyl fentanyl) - 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, and U-51754 at 10 ng/mL spiked concentration. None of the analog-spiked samples screened positive at 1 ng/mL concentration of an analog. With the emergence of new fentanyl analogs and inability of many immunoassays to detect many of them, expansion of LDT LC-MS/MS confirmatory menus or use of broad spectrum LC-HRMS drug screening will likelty be important in clinical laboratories.

3. 6. Clinical performance evaluation

Figure 5 was generated by queryingthe 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 rate with the DRI and the FEN2 assays during this period was 13.3% and 17.3%, respectively, with corresponding LC-MS/MS confirmation rates for immunoassay-positive samples of 88.8% and 96.8% **(Figure 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-positivite rates for DRI and FEN2 in these cohorts were, 11.2% and 3.2% respectively. Higher FP rates for the DRI assay are probably due to its greater susceptibility to inter-individual differences in patient samples (Figure 4) and drug interferences [9]. Estimated false-negativity rates (using 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 **(Figure 5B)**. .

4. Conclusions

LDT LC-MS/MS and LC-QTOF methods employed in this worked allowed objective evaluation of the novel FEN2 assay and its comparison to the previously used DRI assay. The FEN2 assay met the laboratory's performance criteria and correctly classified specimens that were FP and FN by the DRI assay. The LDT mass spectrometry generated data provided objective data demonstrating clear improvement of the FEN2 assay as compared with the DRI assay.. 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.

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.

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.

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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

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

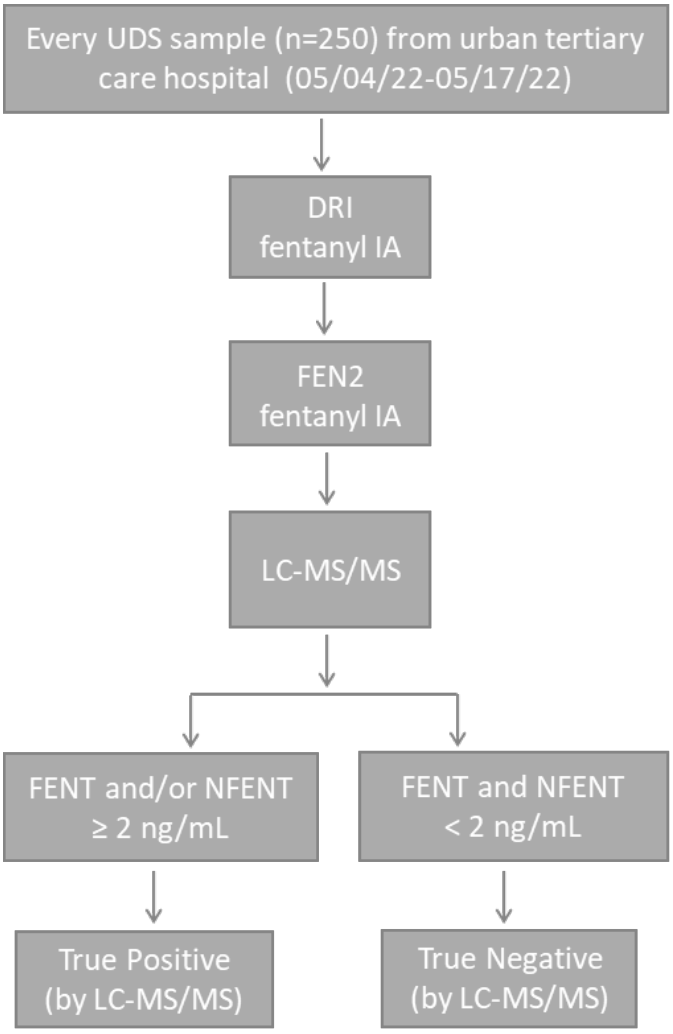


Figure 2. Distribution of fentanyl and norfentanyl concentrations (determined by LC-MS/MS) in the study sample population (n=250)

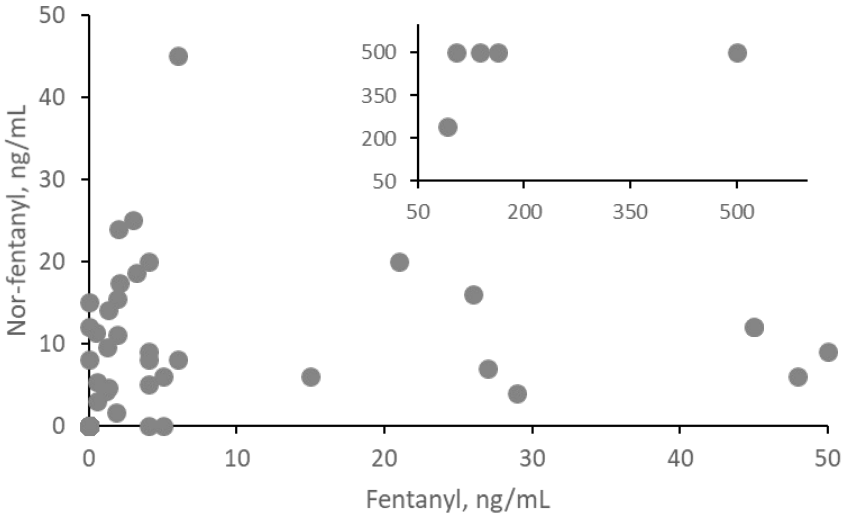


Table 2. Verification of the accuracy (A) and precision (B) of the FEN2 assay (Negative and positive control samples (Neg QC and Pos QC) contained 3.75 and 6.25 ng/mL of norfentanyl, respectively)

LC-MS/MS

A)

FEN2		Negative reference	Positive reference	Total
	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%
Pos QC	0.8%	1.3%	1.5%

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

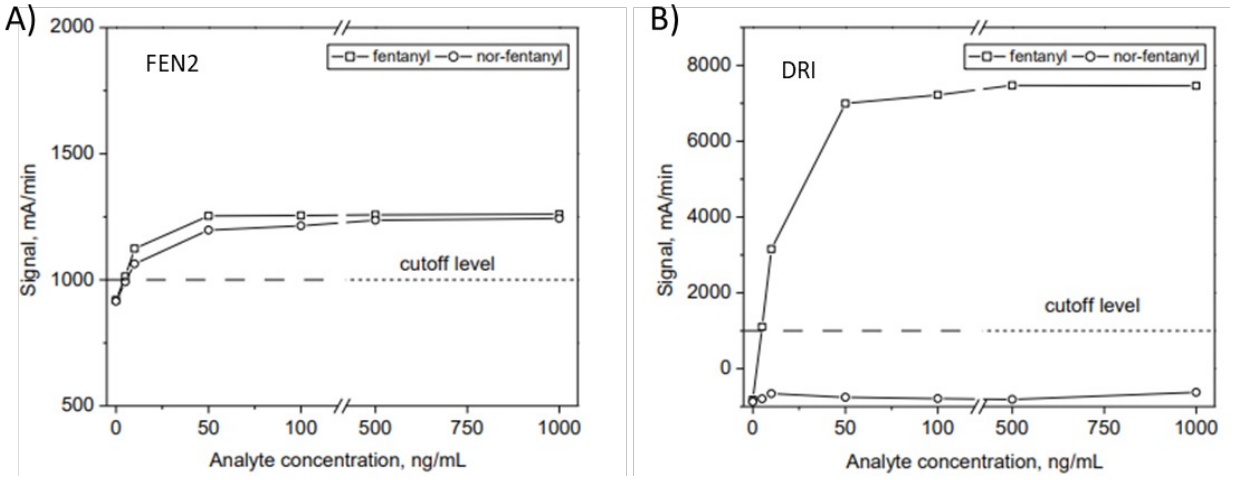


Table 3. Comparison of the clinical performance of the DRI (A) and the FEN2 (B) assays in 250 UDS samples sequentially collected from urban tertiary care hospital (05/04/22-05/17/22). LC-MS/MS was the reference method forcalculating clinical sensitivity and specificity of the assays.

LC-MS/MS

A)

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%

Table 4. A) DRI false-negative samples (among 250 study samples from 05/04/22-05/17/22) and B) hospital services/wards where erroneous DRI screening were obtained from results.

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

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

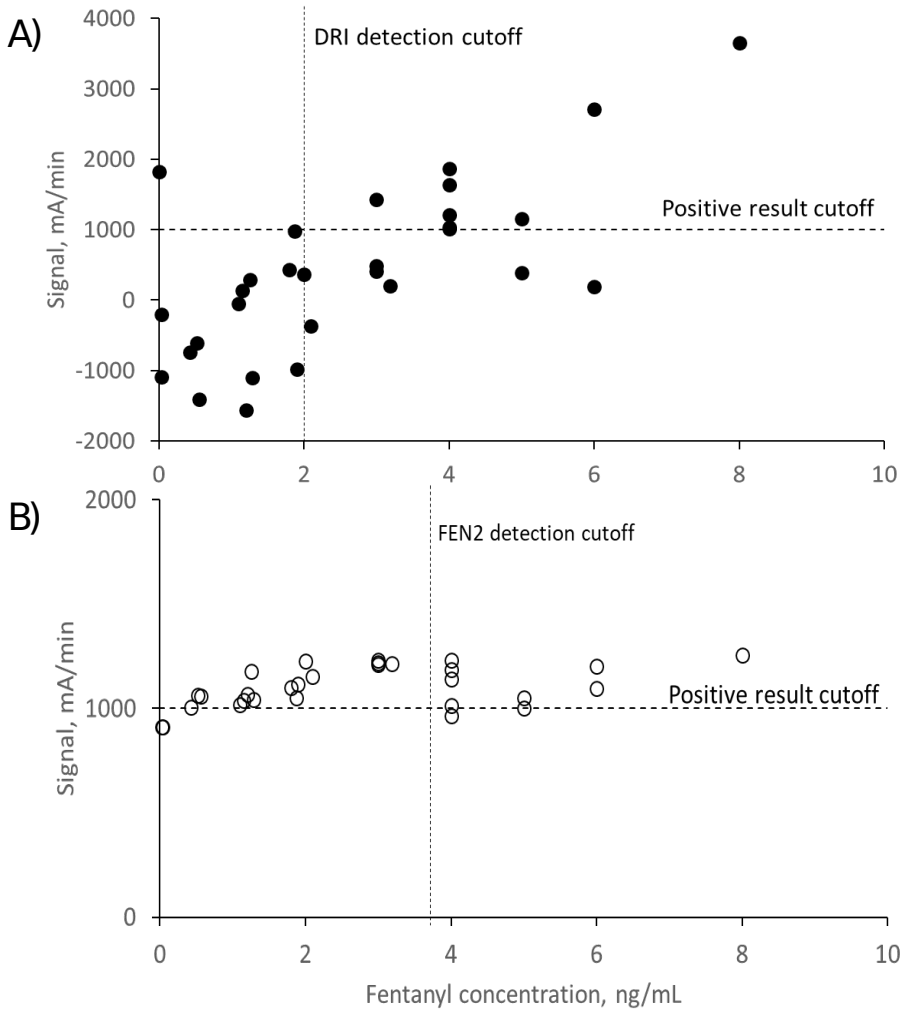
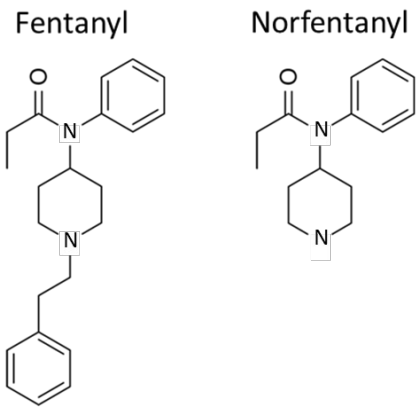


Table 5. Evaluation of the DRI and the FEN2 fentanyl assays' cross-reactivity with the select list of fentanyl analogs

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



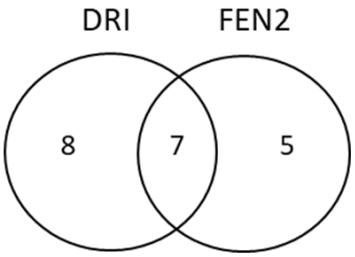


Figure 5. Clinical performance of the DRI and FEN2 assays (data are shown for October 2021 for the DRI and October 2022 for the FEN2). Immunoassay screening and LC-MS/MS confirmation positivity rates (A) and numbers of FPs and FNs (B) for two assays are presented

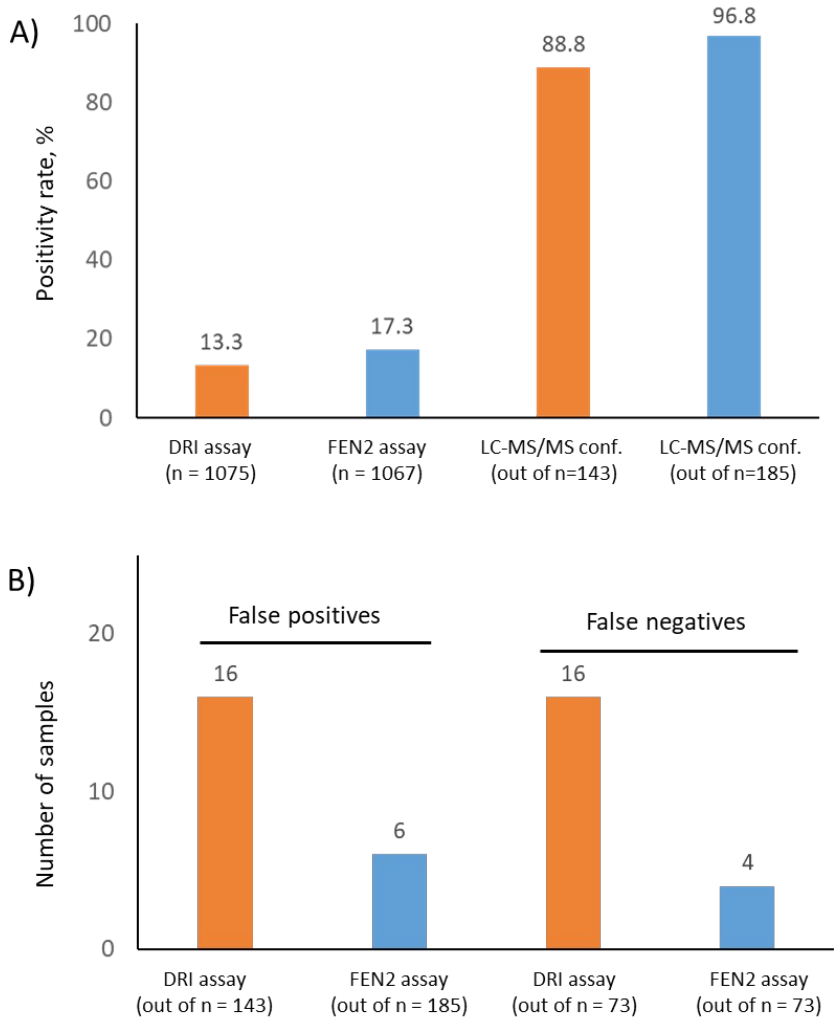
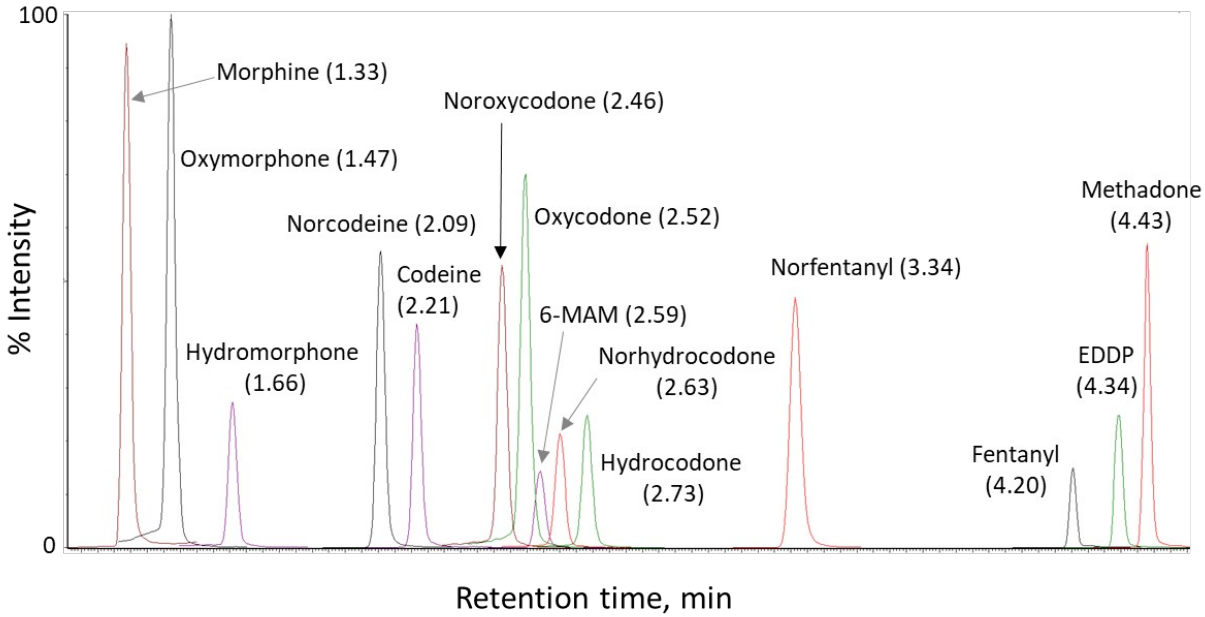


Figure 1S. Extracted ion chromatograms for 14 analytes in the LC-MS/MS confirmatory opiates assay



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703 **Table 1S. Select parameters for the LC-MS/MS confirmatory opiates assay**

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Analyte	Retention time, min	Quantifier* and qualifier transitions	AMR (LLOQ-ULOQ), ng/mL	Precision for 60 days, %CV	
				Low QC	High QC
Morphine	1.33	286.2 > 165.2*, 286.2 > 153.1	100 - 10,000	3.6	3.6
Oxymorphone	1.47	302.1 > 227.1*, 302.1 > 242.1	50 - 5,000	6.5	3.7
Hydromorphone	1.66	286.1 > 157.2*, 286.1 > 153.1	100 - 10,000	5.4	4.5
Norcodeine	2.09	286.2>165.0*, 286.2>181.0	100 - 10,000	4.4	4.0
Codeine	2.21	300.1 > 181.1*, 300.1 > 215.1	100 - 10,000	3.6	3.4
Noroxycodone	2.46	302.1>227.1*, 302.1>187.1	50 - 5,000	4.7	3.9
Oxycodone	2.52	316.1 > 241.2*, 316.1 > 256.2	50 - 5,000	4.5	4.2
6-MAM	2.59	328.1 > 165.1*, 328.1 > 211.2	10 - 1,000	7.4	5.1
Norhydrocodone	2.63	286.2>199.1*, 286.2>171.0	100 - 10,000	7.3	5.2
Hydrocodone	2.73	300.1 > 199.1*, 300.1 >171.1	100 - 10,000	6.1	4.1
Norfentanyl	3.34	233.1 > 84.1*, 233.1 > 56.0	2 – 505	2.9	4.0
Fentanyl	4.20	337.2 >188.2*, 337.2 > 132.2	2 – 505	1.9	3.9
EDDP	4.34	278.2 > 219.2*, 278.2 > 186.2	100 - 3,000	5.9	4.4
Methadone	4.43	310.3 > 223.2*, 310.3 > 219.2	100 - 3,000	3.8	4.2

Table 2S. List of the drugs detected by LC-QTOF with MS^E fragmentation in select DRI-FP samples collected between 10/14/2021 and 01/27/2022

Sample ID	Detected drugs									
FP1	citalopram/ escitalopram	donepezil	memantine	carvedilol	tryptophan	azithromycin				
FP2	gabapentin	diphenydramine	citalopram/ escitalopram	ondansteron-m (8-hydroxy)	zolpidem-m (6-carboxylic acid)	zolpidem				
FP3	citalopram/ escitalopram	donepezil	memantine	carvedilol	tryptophan	azithromycin	viloxazine			
FP4	trimethoprim	sulfamethoxazole	caffeine	setraline	aripiprazole	tryptophan				
FP5	venlafaxine	venlafaxine-m (o-demethyl metabolite)	sitaglipin	metoprolol	naltrexone	1-(3-chlorophenyl) piperazine (mcpp)	trazodone	hydroxy bupropion	theobromine	atorvastatine
FP6	methadone	EDDP	paracetamol	gabapentin	citalopram-m (desmethyl metabolite)	trazodone	diltiazem, deacetyl	metoprolol	metoprolol, hydroxy	1-(3-chlorophenyl) piperazine (mcpp)
FP7	diphenydramine	ketamine	midazolam, alpha-hydroxy	theophyline/ aminophylline	tryptophan	caffeine	midazolam	nicotine	lorazepam	
FP8	metoprolol	metoprolol, hydroxy	metolazone	warfarin	aripiprazole					
FP9	citalopram/escitalo	risperidone, hydroxy	risperidone	cotinine	tryptophan					
FP10	methamphetamine	quetiapine	gabapentin	risperidone, hydroxy	risperidone	caffeine	citalopram/ escitalopram	tryptophan	sertraline	
FP11	diphenydramine	tryptophan	buprenorphine	chlorpheniramine	norbuprenorphine					
FP12	paracetamol	mitragynine-m 7 hydroxy)	ondansteron-m (8-hydroxy)	ondansteron-m (7-hydroxy)	caffeine	theophyline/ aminophylline	tryptophan	diphenydramine		
FP13	benzoylecgonine	cocaine	cocaethylene	ecgonine methyl ester	oxycodone	noroxycodone	fenoterol	meprobamate	alpha-hydroxy flualprazolam	caffeine
FP14	gabapentin	pholedrine (4-hydroxy methamphetamine)	aminoindane (2-AI)	aripiprazole	nicotine	cotinine	cathine (norpseudoephedrine)	tryptophan	aripiprazole-m (dehydro)	topiramate
FP15	diltiazem	quetiapine	diltiazem, deacetyl	metoprolol, hydroxy	metoprolol	lorazepam	atorvastatin	tryptophan		
FP16	benzoylecgonine	metoprolol	ecgonine methyl ester	cocaethylene	tryptophan	cocaine	theophyline/ aminophylline	caffeine	testosterone	
FP17	cotinine	methandrostenolone	olanzapine	tryptophan						
FP18	ciprofloxacin	chloroquine-m (hydroxy metabolite)	gabapentin	metformin	paracetamol	fenoterol	noroxycodone	rizatriptan	lorazepam	sertraline
FP19	levofloxacin	memantine	cetirizine	donepezil	chloroquine-m (hydroxy metabolite)	1-(3-chlorophenyl) piperazine (mcpp)	trazodone	tryptophan	sertraline	
FP20	theobromine	tryptophan	nicotine	levofloxacin						
FP21	diphenydramine	tryptophan	chlorpheniramine, desmethyl	chlorpheniramine						