Using LC-MS/MS LDT to determine fentanyl prevalence and evaluate the FEN2 immunoassay's real-world clinical performance in tertiary care settings

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Background and significance

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].

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 (often times only on physician's request). While LC-MS/MS LDTs 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.

At UCSD Health clinical laboratories we used LC-MS/MS method developed in-house to both systematically assess the prevalence of fentanyl in our UDS samples and evaluate the real-world clinical performance of the novel Roche FEN2 fentanyl immunoassay [3]. The attractive feature of the FEN2 was its low detection cutoff value for norfentanyl (5 ng/mL) compared with other commercially available fentanyl immunoassays. The ability to detect low concentrations of norfentanyl is important due to the short elimination half-life of the parent drug and its extensive metabolism [4]. Continuing rise in the number of overdose deaths from fentanyl (especially from illicitly manufactured fentanyl) makes its quick and reliable detection in clinical settings a pressing issue [5].

Methods and Materials

The in-house opiates LC-MS/MS method was developed and validated according to CLSI guidelines [6]. Fifteen microliters of urine specimens were mixed with beta-glucuronidase in a hydrolysis buffer (IMCS LLC, Irmo, SC) and incubated for 30 minutes 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 QqQ MS with Acquity UPLC (Waters Corporation, Milford, MA) was used for analysis. Samples were separated **(Fig. 1)** on a Waters HSS C18 2.5 um x 2.1 x 150 mm UPLC XP column with UPLC 2.1 mm C18 guard column (Phenomenex, Torrance, CA) using gradient elution over 4.5 minutes. Mobile phase A was 5mM $HCOONH_4$ at pH 3.0 and mobile phase B was 0.1% solution of HCOOH in acetonitrile. 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. Analyte retention times, ion transitions, analytical measurement ranges (AMRs) and precision for opiates LC-MS/MS method are summarized in the **Table 1**

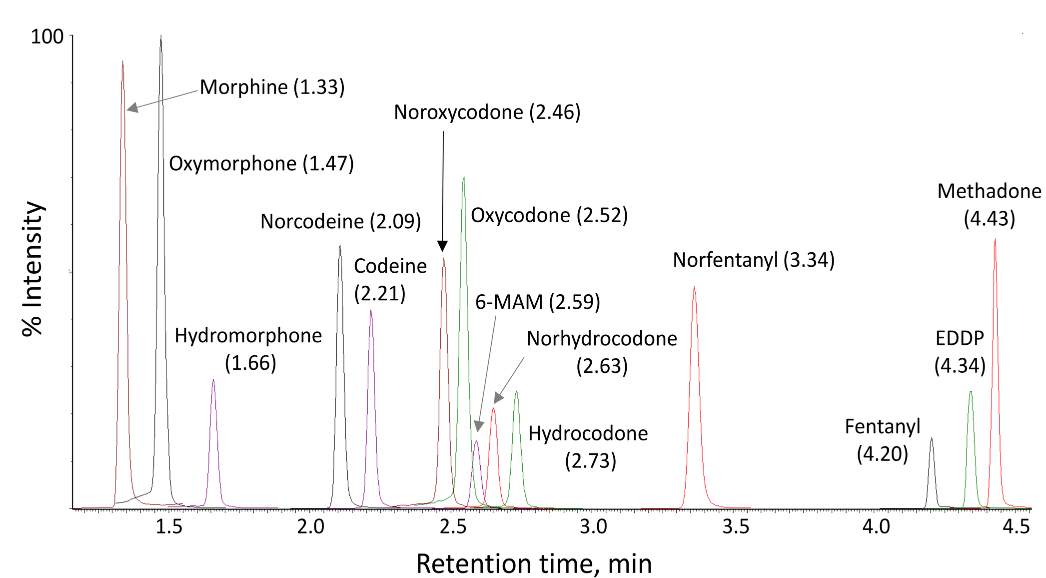

Figure 1. Overlay of EIC for in-house opiates LC-MS/MS method

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 1. Select parameters for in-house opiates LC-MS/MS method

The FEN2 was implemented according to the manufacturer's and its clinical sensitivity and specificity were determined using 250 consecutive random patient specimens **(Fig. 2)** and compared to those of the DRI assay (Thermo Fisher). Real-world clinical performance of the fentanyl immunoassays was evaluated by querying UCSD Health's electronic health records (EHR).

Prevalence of fentanyl in UDS samples in tertiary care hospital

Thirty-eight of the 250 samples were found to contain fentanyl and 49 samples-contained norfentanyl at ≥ 2ng/mL concentration. Fifty-one samples contained fentanyl, norfentanyl, or both analytes at ≥ 2ng/mL, with a median fentanyl and norfentanyl concentration of 5 and 15.5 ng/mL, respectively, and corresponding inter-quartile ranges (IQRs) of 43 and 85 ng/mL. Some samples contained detectable analyte levels below LLOQ.

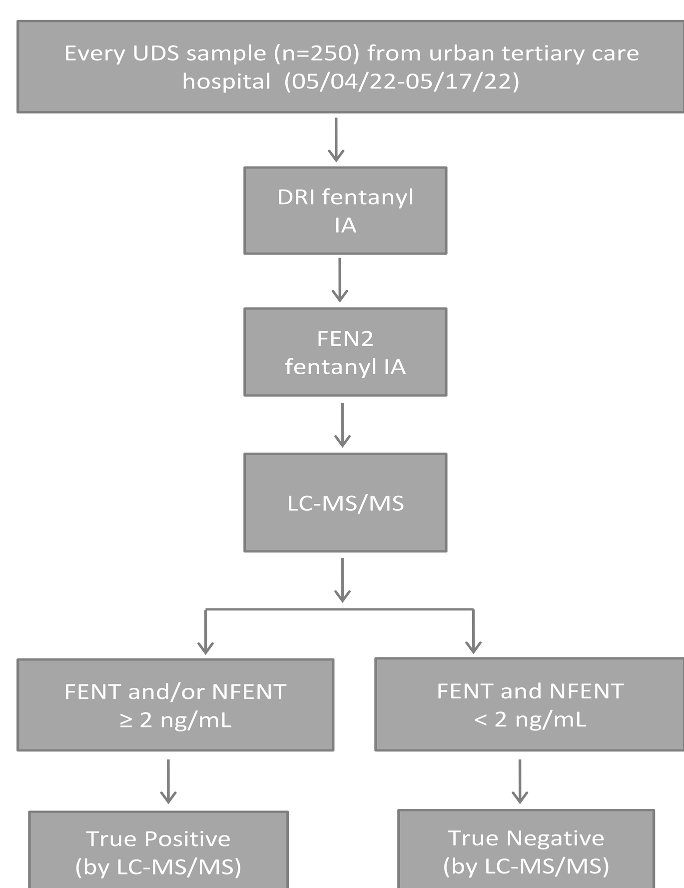

Figure 2. Study design to determine fentanyl prevalence of immunoassay performance

These findings correspond to a 22.8% prevalence of fentanyl in our study population. A previous nation-wide study [7] 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 results 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.

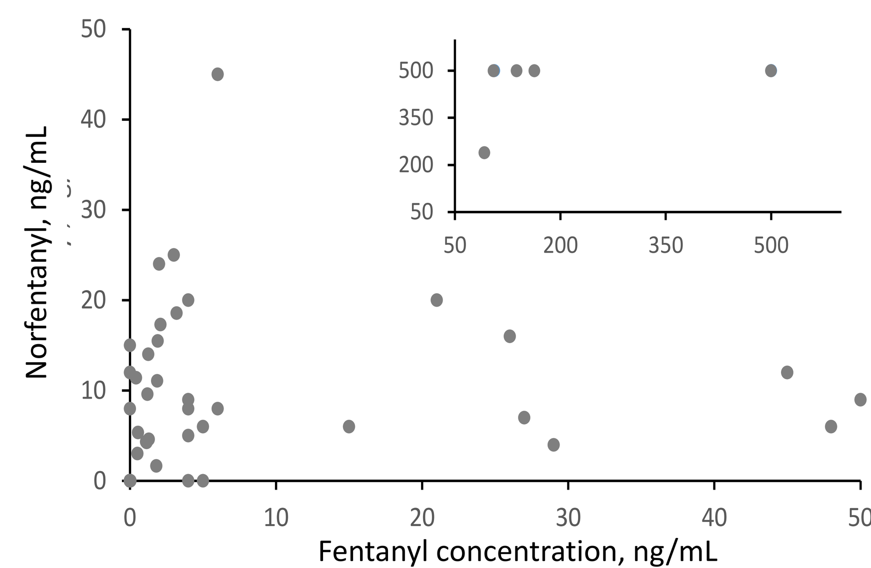

Figure 3. Fentanyl and norfentanyl concentrations (determined by LC-MS/MS) in the study sample population (n=250)

Performance of the FEN2 and DRI assays using study samples

LC-MS/MS

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

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

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

Table 2. Clinical sensitivity and specificity of the fentanyl immunoassays from study samples

	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

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

Table 3. Immunoassay results for select study samples

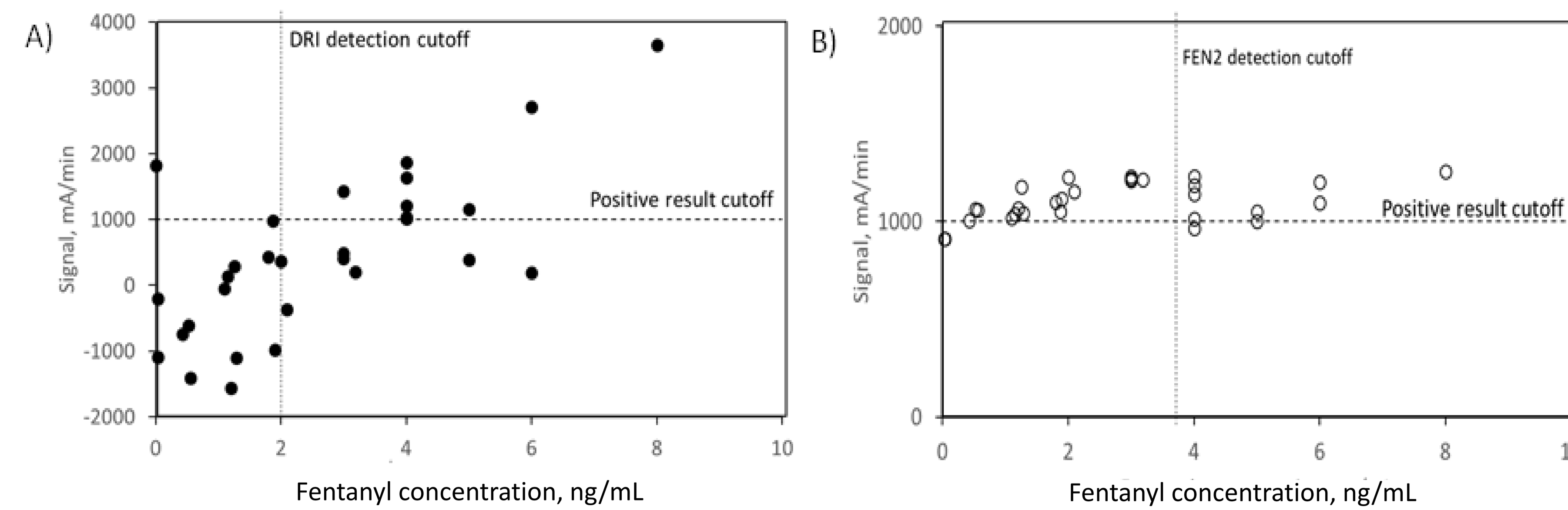

Figure 4. Effect of inter-individual sample differences on the DRI (A) and the FEN2 (B) immunoassay signals (signal≥1000, positive)

Real-world clinical performance of the FEN2 and DRI assays

The overall positivity rates with the DRI and FEN2 assays were 13.3% and 17.3%, respectively, with corresponding LC-MS/MS confirmation rates for immunoassay-positive samples of 88.8% and 96.8% **(Fig. 5A)**. 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. Estimated false-negativity rates (using a smaller subset of total immunoassay-screened samples that were negative on a fentanyl screen, but were reflexed to LC-MS/MS opiates analysis due to positivity on traditional opiate immunoassay screen) were 22% and 5.5% for DRI and FEN2, respectively **(Fig. 5B)**.

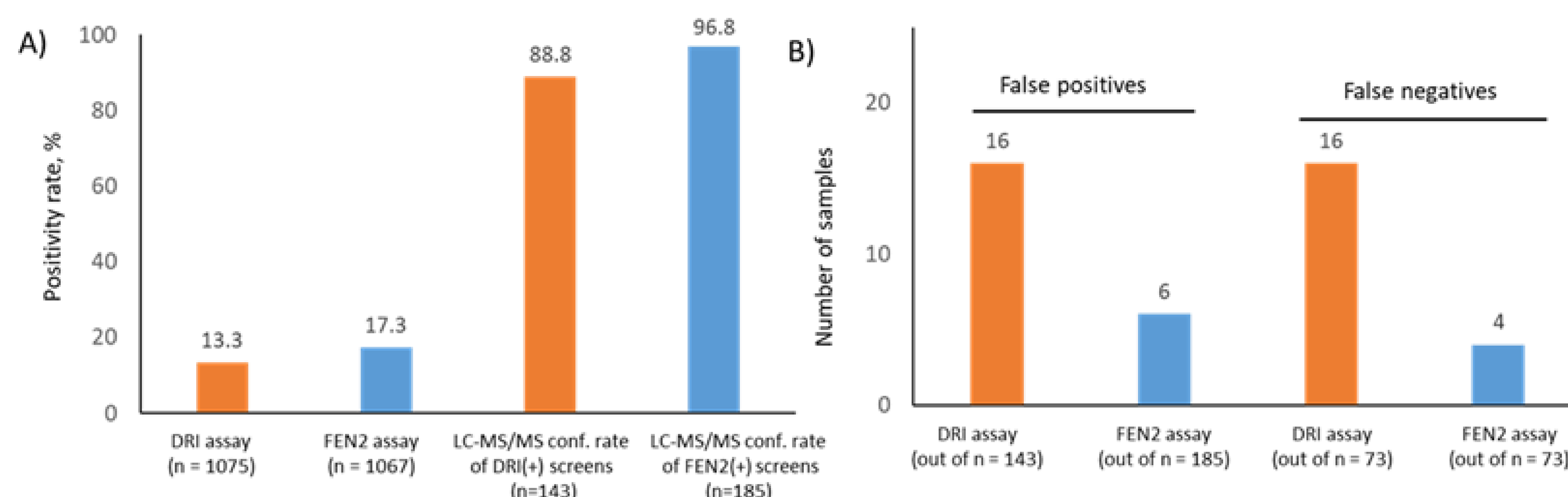

Figure 5. Clinical performance of the DRI and FEN2 assays (data are shown for October 2021 for the DRI and October 2022 for the FEN2). Immunoassay screening and LC-MS/MS confirmation positivity rates (A) and numbers of false positives and false negatives (B) for two assays

Conclusion

LC-MS/MS LDTs in the clinical laboratory are critical not only for the confirmation of presumptively positive UDS samples, but also for clinical implementation and real-world performance evaluation of drug immunoassay. The use of LC-MS/MS LDTs helped us to demonstrated that the FEN2 assay has greater real-world clinical sensitivity and is less prone to false-positives than the DRI assay. These findings support the use of FEN2 in routine clinical practice for clinical toxicology testing.

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