RAPID HOMOGENEOUS IMMUNOASSAY FOR THE 1 NG/ML DETECTION OF FENTANYL AND NORFENTANYL IN URINE

#B-316

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BACKGROUND

Fentanyl is a synthetic opioid analgesic that is 50 to 100 times more potent than morphine. Originally developed for chronic pain management and postoperative analgesia, fentanyl is available in various formulations, including intravenous anesthesia (Sublimaze®), transdermal patches (Duragesic®), and transmucosal lozenges (Actiq®). Therapeutic doses range from 2.5–10 mg in Duragesic® and 0.2–1.6 mg in Actiq®. However, fentanyl has become a major contributor to the ongoing opioid epidemic, with illicitly manufactured fentanyl (IMF) driving a sharp rise in overdose deaths. Illicit fentanyl is frequently mixed with heroin, counterfeit pills, and other drugs, significantly increasing the risk of fatal overdoses. Street names for illicit fentanyl include Apache, China White, Jackpot, and Tango. Fentanyl has a half-life of 3 to 12 hours and undergoes extensive metabolism via N-dealkylation and hydroxylation, with over 90% of the dose eliminated as norfentanyl and hydroxylated metabolites. Less than 7% is excreted unchanged in urine. The increasing prevalence of fentanyl has heightened the demand for highly sensitive and specific detection methods. ARK Diagnostics has developed a new fentanyl assay, designed to detect fentanyl at a cutoff of 1 ng/mL with at least 90% cross-reactivity to norfentanyl. This high sensitivity assay with minimal cross reactivity to other structurally related compounds makes the assay a valuable tool for clinical and forensic toxicology laboratories, aiding in the detection of fentanyl use and supporting efforts to address the ongoing public health crisis.

METHODS

This new fentanyl assay is a liquid stable homogeneous enzyme immunoassay, consisting of two reagents, with a cutoff of 1 ng/mL fentanyl. The performance of this assay was evaluated on the Beckman Coulter AU480 Automated Clinical Chemistry Analyzer, including precision, specificity to the major metabolite norfentanyl and fentanyl analogs, analytical recovery of fentanyl and norfentanyl mixtures, and method comparison with LC-MS/MS.

RESULTS

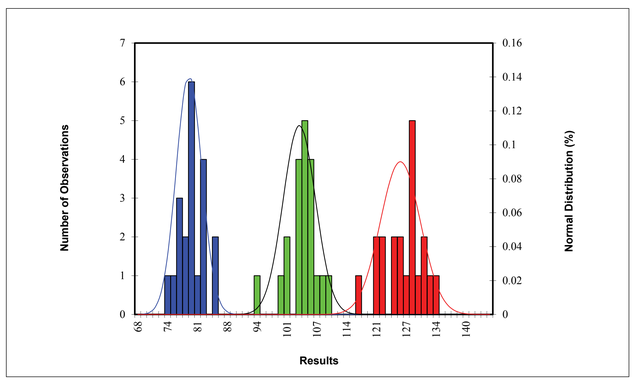
PRELIMINARY PRECISION

Pooled human urine was spiked with fentanyl to achieve concentrations at ±25% and ±50 increments from the cutoff calibrator (1 ng/mL). Twenty (20) replicates of each sample were assayed in qualitative mode. The 1 ng/mL cutoff is assigned as 100 normalized units, such that qualitative results ≥100 are positive and qualitative results <100 are negative.

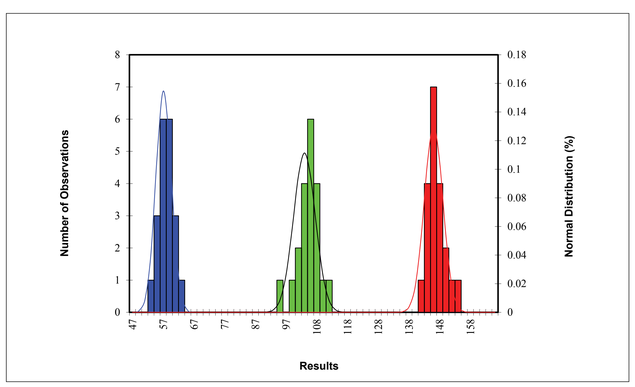
Fentanyl (ng/mL)	Cutoff (%)	Mean (Normalized Units)	SD	CV (%)
0.50	-50	56.9	2.6	4.5
0.75	-25	79.2	2.8	3.6
1.00	Cutoff	103.5	3.6	3.5
1.25	+25	125.8	4.4	3.5
1.50	+50	146.3	3.1	2.1

HISTOGRAM OVERLAP ANALYSIS (QUALITATIVE ANALYSIS)

Histogram analysis shows the distribution of fentanyl values for each sample. Twenty replicates each of the ±25% controls (0.75 ng/mL and 1.25 ng/mL), ±50% controls (0.50 ng/mL and 1.50 ng/mL), and the 1.0 ng/mL cutoff calibrator were tested together in a single run. The resulting distributions were distinct with no overlap.



±25% Control Precision vs 1.0 ng/mL Cutoff Calibrator

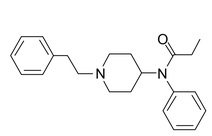


±50% Control Precision vs 1.0 ng/mL Cutoff Calibrator

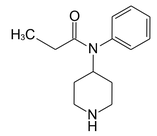
SPECIFICITY

Major Metabolite Norfentanyl

The major metabolic pathway of fentanyl in humans involves piperidine N-dealkylation and results in the major metabolite norfentanyl. Two minor metabolic pathways result in minor metabolites despropionylfentanyl and hydroxyfentanyl (and later hydroxynorfentanyl) (Labroo et al.). More than 90% of the dose is eliminated as norfentanyl and hydroxylated metabolites. Less than 7% of the dose is excreted unchanged in urine.[1] Cross-reactivity to the major metabolite norfentanyl is desirable for the detection and monitoring of fentanyl drug use, since norfentanyl is detectable in the urine for a considerably longer period of time than the parent drug fentanyl.[2]



Fentanyl



Norfentanyl

This new assay demonstrated 90.9% cross-reactivity to norfentanyl, corresponding to an approximate equivalent cutoff of 1.1 ng/mL relative to the 1 ng/mL fentanyl cutoff.

Compound	Concentration Approximately Equivalent to the Cutoff (ng/mL)	Percent Cross-reactivity (%)
Norfentanyl	1.1	90.9

Fentanyl and norfentanyl are seldom detected separately in urine; instead, they're typically found together, with norfentanyl levels reported as up to 15 times higher than fentanyl.[3] The table below illustrates the assay's response with 30 samples containing mixtures of fentanyl and norfentanyl spiked at varying concentrations ranging from 0 – 1.0 ng/mL fentanyl and 0 – 1.25 ng/mL norfentanyl in negative human urine. As shown in the table, many samples with fentanyl concentrations below 1 ng/mL tested positive using the new fentanyl assay, reflecting its increased sensitivity when norfentanyl is present.

Fentanyl/Norfentanyl Mixtures		Norfentanyl (ng/mL)					
		0.00	0.25	0.50	0.75	1.00	1.25
Fentanyl (ng/mL)	0.00	neg	neg	neg	neg	neg	**POS**
	0.25	neg	neg	neg	**POS**	**POS**	**POS**
	0.50	neg	neg	**POS**	**POS**	**POS**	**POS**
	0.75	neg	**POS**	**POS**	**POS**	**POS**	**POS**
	1.00	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**

Metabolites and Structural Analogs of Fentanyl

The following metabolites and structural analogs of fentanyl were approximately equivalent to the 1 ng/mL cutoff at the concentrations tested with the fentanyl assay.

Compound	Concentration Approximately Equivalent to the Cutoff (ng/mL)	Percent Cross-reactivity (%)
Acetyl fentanyl	0.98	102.0
Acrylfentanyl	1.02	97.6
Isobutyryl fentanyl	1.04	96.3
Butyryl fentanyl	1.04	95.8
4-Fluoro-isobutyryl fentanyl	1.06	94.8
Acetyl norfentanyl	1.06	94.6
Para-fluoro fentanyl	1.11	89.7
Valeryl fentanyl	1.15	87.0
Furanyl fentanyl	1.20	83.6
Para-fluorobutyryl fentanyl (p-FBF)	1.23	81.0
Ocfentanil	1.29	77.6
β-hydroxyfentanyl	7.97	12.5
(±) β-hydroxythiofentanyl	23.89	4.2
Norcarfentanil	49.02	2.0
(±)-3-cis-methyl fentanyl	79.01	1.3
Despropionyl fentanyl (4-ANPP)	246.51	0.4
Carfentanil	512.07	0.2
Sufentanil	1,454.2	0.07
Remifentanil	>10,000	<0.01
Alfentanil	>100,000	<0.001

Structurally Unrelated Compounds

No interference was observed with the following 92 structurally unrelated compounds tested at a minimum concentration of 50,000 ng/mL.

Compound			
(-) pseudoephedrine	clomipramine	imipramine	1procainamide
(+) pseudoephedrine	clonidine	ketamine	procyclidine
(+/-) methamphetamine	cocaine	levofloxacin	promethazine
(S) naproxen	cotinine	lidocaine	Quinacrine
1R-2S(-) ephedrine	cyclobenzaprine	lorazepam	quinidine
1S-2R(-) ephedrine	delta 9-carboxy-tetrahydro cannabinol	lormetazepam	quinine
9-OH-risperidone	desipramine	LSD	rifampicin
acetaminophen	diazepam	maprotiline	ritalinic acid
acetylsalicylic acid	digoxin	methaqualone	salicylic acid
amitriptyline	diphenhydramine	methylphenidate	scopolamine
amobarbital	d-methamphetamine	metronidazole	secobarbital
amoxapine	doxepine	mitragynine	sertraline
amoxicillin	ecgonine	NAPA	sildenafil
benzoylecgonine	enalapril	nifedipine	thioridazine
brompheniramine	ethyl B-D glucuronide	nordiazepam	tilidine
bupropion	fenfluramine	oxazepam	trazodone
butabarbital	fluoxetine	PCP	trimethoprim
cannabinol	fluphenazine	pentobarbital	triprolidine
captopril	glutethimide	perphenazine	tyramine
carbamazepine	hydromorphone β-D-3 glucuronide	phenelzine	verapamil
chlordiazepoxide	hydroxyzine	phenobarbital	xylazine
chlorpromazine	ibuprofen	phenylpropanolamine	zidovudine (AZT)
cimetidine	imipramine	phenytoin	zolpidem

METHOD COMPARISON

A total of 100 unaltered, de-identified clinical urine specimens were analyzed for fentanyl with the fentanyl assay and by LC-MS/MS. The LC-MS/MS confirmatory method was performed by a licensed reference laboratory and used a fentanyl cutoff of 0.2 ng/mL and norfentanyl cutoff of 1 ng/mL.

The fentanyl assay demonstrated 100% sensitivity (true positive rate) and 100% specificity (true negative rate) relative to LC-MS/MS. Twenty (20) specimens with LC-MS/MS values <1 ng/mL fentanyl tested positive due to the presence of norfentanyl in the specimens. Results are summarized.

		LC-MS/MS (+)	LC-MS/MS (-)	Sensitivity	Specificity
Fentanyl Immunoassay	(+)	50	0	100%	100%
	(-)	0	50		

Fentanyl Immunoassay Result	Low Negative <50% of cutoff by LC-MS/MS (<0.5 ng/mL)	Near Cutoff Negative (Between 50% below the cutoff and the cutoff by LC-MS/MS) (0.5 – 0.9 ng/mL)	Near Cutoff Positive (Between the cutoff and 50% above by LC-MS/MS) (1.0 – 1.5 ng/mL)	High Positive (Greater than 50% above the cutoff by LC-MS/MS) (>1.5 ng/mL)
Positive	1*	19*	7	23
Negative	50			

*Twenty (20) specimens with LC-MS/MS values <1 ng/mL fentanyl tested positive due to the presence of norfentanyl in the specimens.

Specimens with LC-MS/MS values <1 ng/mL fentanyl

Sample #	LC-MS/MS (ng/mL)		Immunoassay POS/NEG
	Fentanyl	Norfentanyl	
1	0.7	2.1	POS
2	0.4	7.6	POS
3	0.9	63.4	POS
4	0.6	16.9	POS
5	0.6	3.7	POS
6	0.9	12.3	POS
7	0.5	7.9	POS
8	0.9	62.6	POS
9	0.5	425.4	POS
10	0.9	6.5	POS
11	0.6	14.5	POS
12	0.9	2.2	POS
13	0.8	15.9	POS
14	0.5	5.2	POS
15	0.7	3.1	POS
16	0.6	161.7	POS
17	0.6	19	POS
18	0.6	13.8	POS
19	0.8	45.8	POS
20	0.6	14.6	POS

CONCLUSIONS

The new assay provides a highly sensitive and rapid method for detecting fentanyl and its major metabolite, norfentanyl, in human urine. With at least 90% cross-reactivity to norfentanyl, the assay improves the detection of fentanyl exposure. Its rapid performance and compatibility with a wide range of clinical chemistry analyzers make it a dependable tool for both clinical and forensic toxicology applications.

REFERENCES

1. Labroo, RB et al. 1997. Fentanyl metabolism by human hepatic and intestinal cytochrome p450 3A4: Implications for interindividual variability in disposition, efficacy, and drug interactions. Drug Metab Dispos. 25(9):1072-80.
2. Silverstein, J. H. et al. 1993. An analysis of the duration of fentanyl and its metabolites in urine and saliva. Anesth Analg. 76:618-621.
3. Wu F, Slawson MH, Johnson-Davis KL. Metabolic Patterns of Fentanyl, Meperidine, Methylphenidate, Tapentadol and Tramadol Observed in Urine, Serum or Plasma. J Anal Toxicol. 2017 May 1;41(4):289-299.

REGULATORY STATUS

Product under development. Not FDA cleared for sale in the U.S.