Immunoassay-Based Detection of Fentanyl Analogs in Forensic Toxicology

Madison Schackmuth, BS* and Sarah Kerrigan, PhD

Department of Forensic Science, Sam Houston State University, Huntsville, TX 77340

INTRODUCTION

- Scheduling and legislative actions rarely keep pace with clandestine development
- Structural modifications are primarily observed at the *N*-acyl group, the phenethyl group, and on the piperidine ring [1]
- The majority of toxicology laboratories employ immunoassay-based screening techniques [2]
- Due to their small size, bioconjugation techniques are used to attach the target drug to larger carrier protein
- The nature of the covalent attachment can influence the overall specificity of the antibody reagent, and therefore assay utility

MATERIALS & METHODS

Fourteen fentalogs were purchased from Cerilliant (Round Rock, TX). Two ELISA kits were obtained from Randox Laboratories Ltd. (Kearnesysville, WV): Fentanyl ELISA Plate and Carfentanil/Remifentanil ELISA Plate. The Fentanyl Group Kit and Fentanil Group Forensic Kit were obtained from Neogen (Lansing, MI). The Fentanyl ELISA Kit was obtained from Immunalysis (Pomona, CA). A Direct-Q3 system (Millipore, Billerica, MA) was used to obtain deionized water. A Biotek ELx50/8 Microplate Strip Washer (Winooski, TX) and a Dynex Technologies Opsys MR Plate Reader (Chantilly, VA) were used to perform all assays.

Assays were performed in accordance with manufacturers' recommendations (Table 1). Both Randox kits required a 1:4 dilution of urine samples with wash buffer prior to analysis. The absorbance was measured ($A_{450\text{-}630\ nm}$) following the addition of the acidic stop solution. Hydrochloric acid (1N) was used as the stop solution in the Neogen Fentanil Group Kit in place of the provided Red Stop Solution as a 650 nm filter was not available.

Dose-response curves were generated over an extended range of concentrations (0.08 – 50 ng/mL) by plotting the percent binding (%B) against the logarithm of the concentration (C). Cross-reactivity was defined as $(C_{cutoff})x100/(C_{equiv})$, where (C_{equiv}) was the equivalent concentration of fentalog required to produce the same response as the target drug at the specified cutoff. Cross-reactivities were evaluated at three levels: at the effective concentration to achieve fifty percent binding (EC_{50}) of the target analyte, 1 ng/mL (the recommended cutoff concentration in urine for drug impaired driving casework [2]) and 0.5 ng/mL.

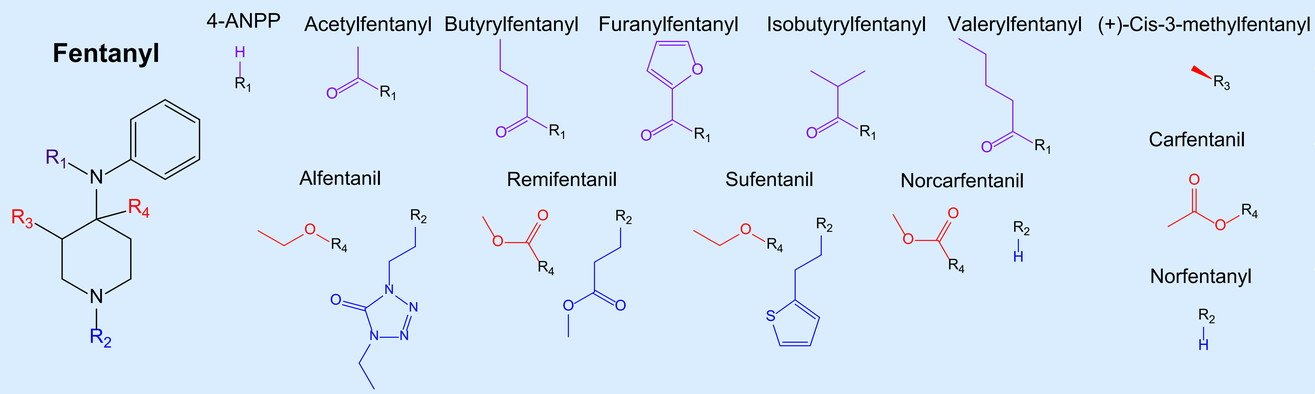
Table 1: Manufacturer Recommendations.

Manufacturer	Target Drug	Urine (µL)	Conjugate (µL) / Time[a] (min)	TMB[b] (µL) / Time[a] (min)	Stop (µL)
Randox	Norfentanyl	50	75/60	125/20	100
Randox	Carfentanil/ Remifentanil	50	100/60	125/20	100
Neogen Fentanyl	Fentanyl	20	100/45	100/30	100
Neogen Fentanil	Alfentanil	20	180/45	120/30	50
Immunalysis	Fentanyl	20	100/60	100/30	100

[a]Incubation time [b]TMB = tetramethylbenzidine

RESULTS & DISCUSSION

Figure 1: Fentalog Structures



Tables 2 & 3: Cross-reactivity Data

Analog	Modification	Cross-reactivity (%) Randox Carfentanil/Remifentanil MF	EC_{50}	0.5 ng/mL	1 ng/mL	Cross-reactivity (%) Neogen Fentanil MF	EC_{50}	0.5 ng/mL	1 ng/mL	Cross-reactivity (%) Randox Fentanyl MF	EC_{50}	0.5 ng/mL	1 ng/mL	Cross-reactivity (%) Neogen Fentanyl MF	EC_{50}	0.5 ng/mL	1 ng/mL	Cross-reactivity (%) Immunalysis Fentanyl MF	EC_{50}	0.5 ng/mL	1 ng/mL
Norfentanyl	Phenethyl	NR	<1	<1	<2	<1	<1	<1	<2	**100**	**100**	**100**	**100**	<1	<1	<1	<2	<1	<1	<1	<2
Fentanyl	-	<1	<1	<1	<2	<5	<5	<5	<10	790	720	250	333	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
(+)-Cis-3-methylfentanyl	Piperidine	<1	<1	<1	<2	<1	<1	<1	<2	31	<5	50	25	50	3	13	4	NR	9	<5	<10
Carfentanil	Piperidine	162	115	111	91	88	324	>1600	435	<1	<1	<1	<2	6	5	25	6	NR	<1	<1	<2
Alfentanil	Piperidine/Phenethyl	30	38	38	<20	**100**	**100**	**100**	**100**	<1	<1	<1	<2	<1	<1	<1	<2	NR	<1	<1	<2
Sufentanil	Piperidine/Phenethyl	13	15	<10	<20	270	524	>1600	625	<1	<1	<1	<2	<1	<1	<1	<2	NR	<1	<1	<2
Remifentanil	Piperidine/Phenethyl	**100**	**100**	**100**	**100**	76	110	200	185	<1	<5	<5	<10	<1	<1	<1	<2	NR	<1	<1	<2
Norcarfentanil	Piperidine/Phenethyl	<5	91	91	71	NR	17	15	19	NR	<5	<5	<10	NR	<1	<1	<2	NR	<1	<1	<2
Isobutyrylfentanyl	*N*-acyl	NR	<1	<1	<2	<1	<1	<1	<2	NR	144	143	119	66	104	102	101	NR	88	68	53
Acetylfentanyl	*N*-acyl	NR	<1	<1	<2	<1	<1	<1	<2	37	<5	50	33	42	57	83	83	NR	277	161	111
Valerylfentanyl	*N*-acyl	NR	<1	<1	<2	<1	<1	<1	<2	NR	60	67	67	208	81	71	80	NR	124	114	71
4-ANPP	*N*-acyl	NR	<1	<1	<2	<1	<1	<1	<2	<1	<1	<1	<2	<1	<1	2	<2	NR	<5	<5	<10
Furanylfentanyl	*N*-acyl	NR	<1	<1	<2	<1	<1	<1	<2	NR	400	357	333	180	100	98	100	NR	212	156	111
Butyrylfentanyl	*N*-acyl	NR	<1	<1	<2	<1	<5	<5	<10	NR	450	250	294	96	85	111	77	NR	164	119	77

MF, Manufacturer; NR, Not Reported.

Figure 2: Neogen Fentanyl Dose-Response Curve

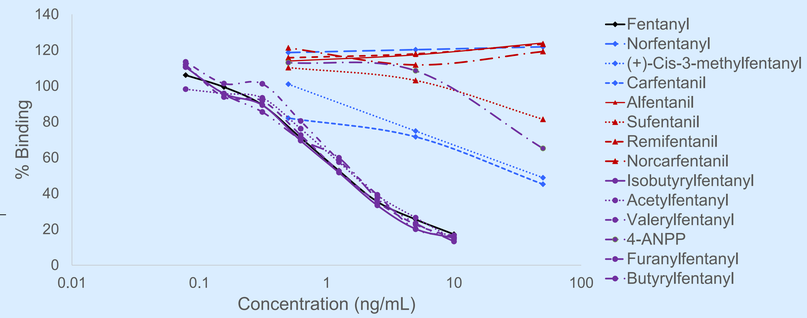


Figure 1: Fentanyl and analogs (above).

Tables 2 & 3: Cross-reactivity of ELISA kits towards fentanyl analogs. The target compound for each assay is shown in bold. The EC50 for the target compounds in the Randox Carfentanil/Remifentanil, Randox Fentanyl, Neogen Fentanyl, Neogen Fentanil, and Immunalysis assays were 0.3, 3.6, 1.3, 1.1, and 0.4 ng/mL, respectively.

Figure 2: Dose-response Curve (left).

- The Immunalysis Fentanyl, Randox Fentanyl, and Neogen Fentanyl Group ELISAs were effective detecting analogs modified at the *N*-acyl group (R_1)
- 4-ANPP was not highly cross-reactive with any of the assays evaluated
- The Randox Fentanyl ELISA was the only assay with high cross-reactivity towards both fentanyl and norfentanyl, and may be preferable if both blood and urine are to be analyzed
- Randox Carfentanil/Remifentanil and Neogen Fentanil Group kits were effective detecting analogs modified on both the piperidine ring (R_4) and at the phenethyl group (R_2)
- Randox Carfentanil/Remifentanil ELISA was highly specific for target compounds and carfentanil metabolite

CONCLUSIONS

- No single assay had sufficient cross-reactivity to identify all of the fentalogs tested
- Multiple ELISAs would be required to effectively screen for a broad range of modifications at $R_{1\text{-}4}$
- Purchasing multiple immunoassays for fentalog screening is inefficient
- Alternative MS-based screening for fentalogs and other new psychoactive substances may be preferred

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[2] B.K. Logan, A.L. D'Orazio, A.L.A. Mohr, J.F. Limoges, A.K. Miles, C.E. Scarneo, S. Kerrigan, L.J. Liddicoat, K.S. Scott, M.A. Huestis (2018) Recommendations for toxicological investigation of drug-impaired driving and motor vehicle fatalities-2017 Update. J Anal Toxicol 42(2) 63-68.

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