Immunoassay-based detection of fentanyl analogs in forensic toxicology

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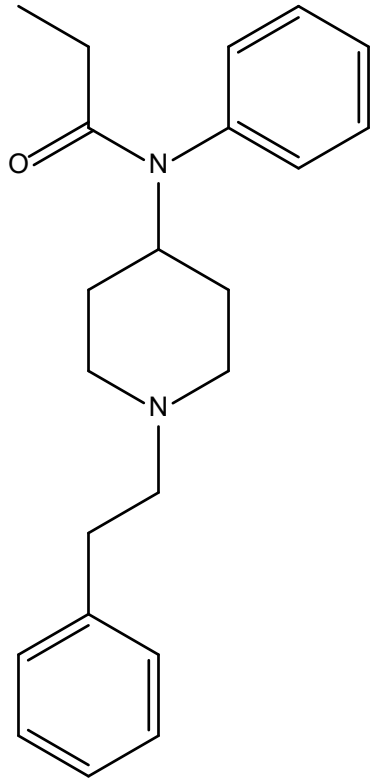
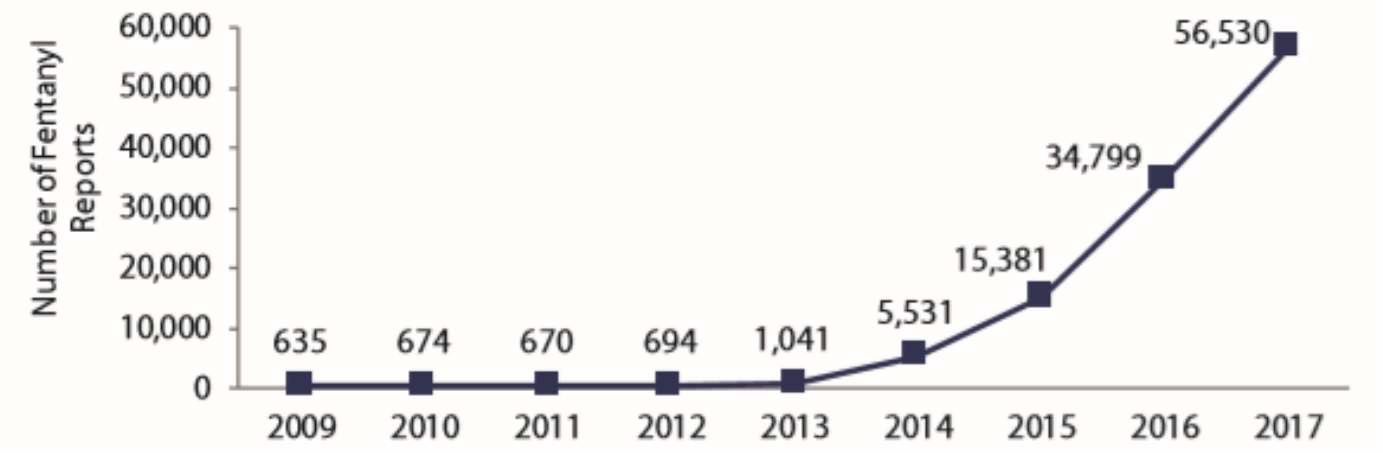
Introduction

- Fentanyl and its analogs are synthetic opioid μ receptor agonists
  - Cause severe respiratory depression
  - Transdermal absorption
- Known potencies substantially higher than morphine
  - remifentanil (x300), sufentanil (x1000), and carfentanil (x10000)
- Several fentanyl analogs have legitimate anesthetic/analgesic use

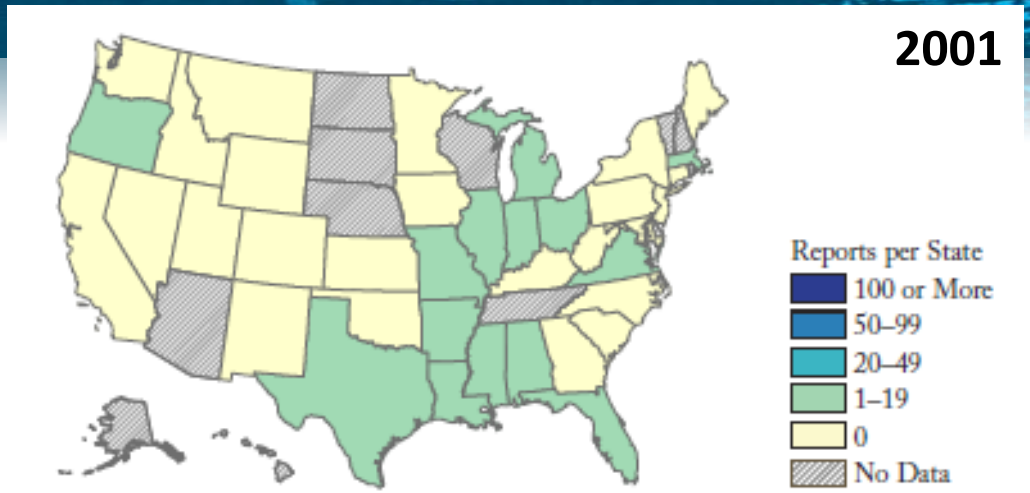


Surveillance Systems

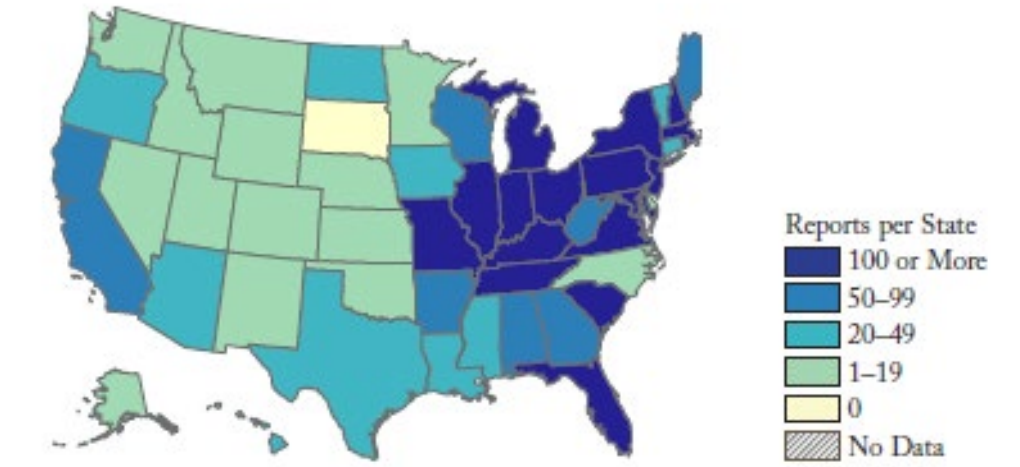
- Global
  - United Nations Office on Drugs and Crime (UNODC)
- International
  - European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)
- National
  - National Forensic Laboratory Information System (NFLIS)



2001



2015



NFLIS Special Maps Release 2019 NFLIS Brief: Fentanyl, 2001-2015

Background

- 29.5 million people globally suffer from drug abuse disorders
  - Opioids account for 70% of negative health impacts (UNODC, 2017)
- Analogs identified as adulterants in ecstasy, hydrocodone, heroin, and crack cocaine
- Drug Enforcement Administration (DEA) ongoing scheduling

Table 2.1 *NARCOTIC ANALGESICS*
Number and percentage of narcotic analgesic reports in the United States, January 2018–June 2018[1]

Narcotic Analgesic Reports	Number	Percent
Fentanyl	37,140	43.00%
Oxycodone	13,351	15.46%
Buprenorphine	8,921	10.33%
Hydrocodone	7,997	9.26%
Tramadol	3,413	3.95%
Acetyl fentanyl	2,246	2.60%
Morphine	2,014	2.33%
Codeine	1,380	1.60%
Methadone	1,230	1.42%
Hydromorphone	1,229	1.42%
ANPP	891	1.03%
Fluoroisobutyryl fentanyl	876	1.01%
Cyclopropyl fentanyl	852	0.99%
Carfentanil	661	0.77%
Methoxyacetyl fentanyl	603	0.70%
Other narcotic analgesics	3,562	4.12%
Total Narcotic Analgesic Reports[2]	86,365	100.00%
Total Drug Reports	772,078	

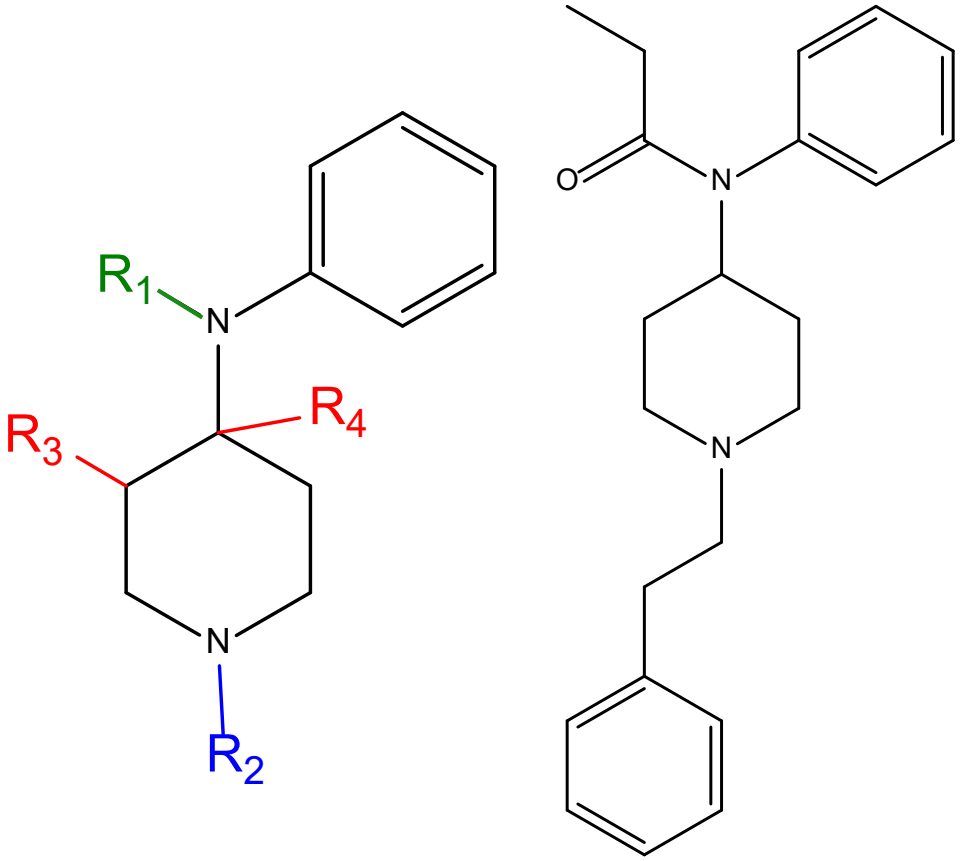
ANPP=4-Anilino-N-phenethyl-4-piperidine

NFLIS Drug 2018 MY Report

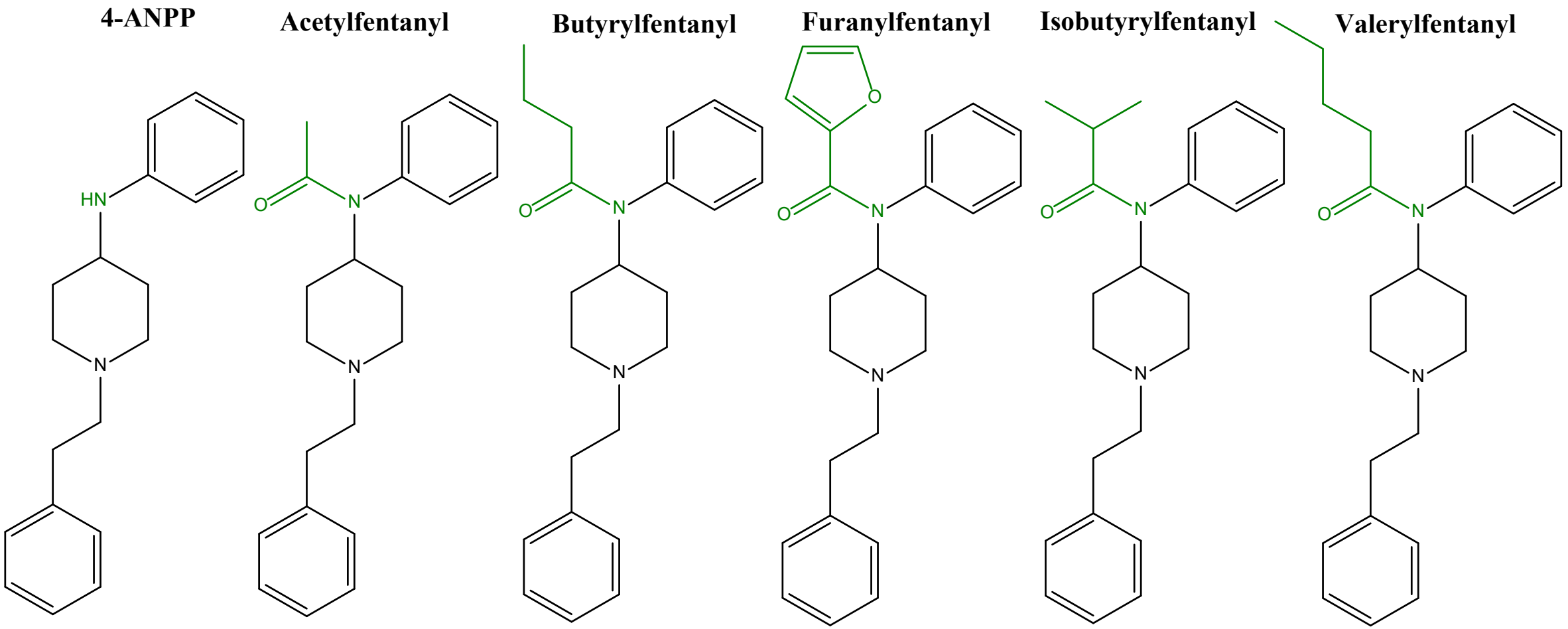
Fentanyl Analogs (*Fentalogs*)

- Fentanyl, *N*-(1-(2-phenethyl)-4-piperidinyl-*N*-phenyl-propanamide

- Fentalogs include structural alterations at:
  - *N*-acyl group (R_1)
  - Phenethyl group (R_2)
  - Piperidine ring (R_3, R_4)
  - Combinations thereof

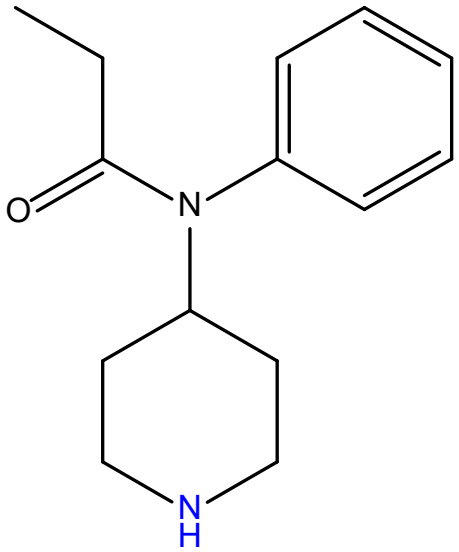


Six *N*-acyl substituted Fentalogs (R_1)



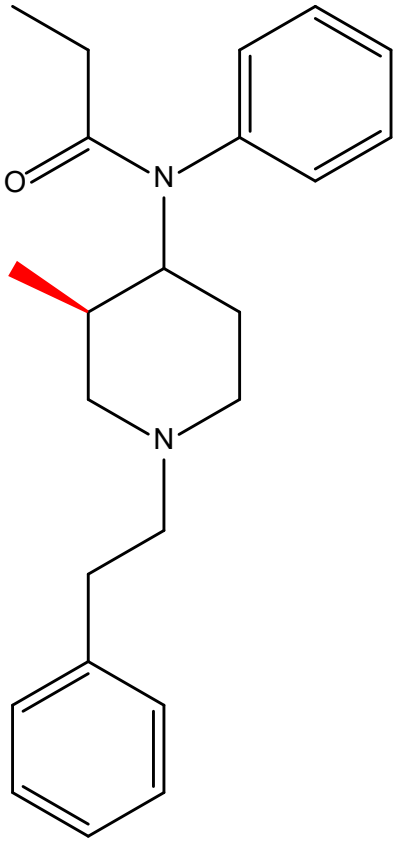
One Phenethyl substituted Fentalog (R_2)

Norfentanyl

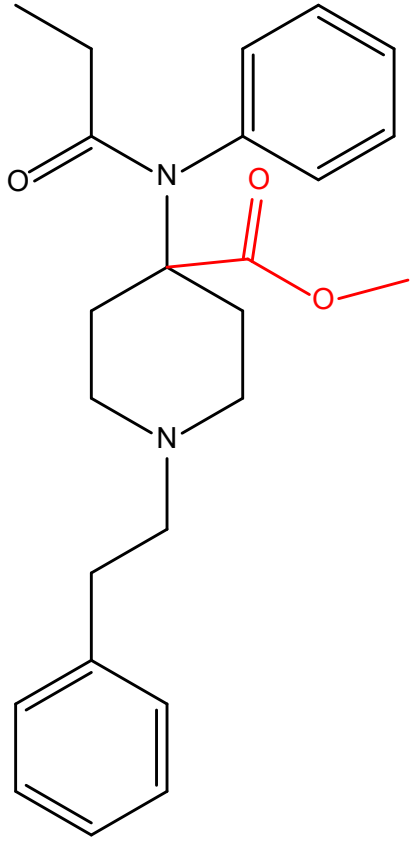


Two Piperidine substituted Fentalogs (R_3 & R_4)

(+)-Cis-3-methylfentanyl

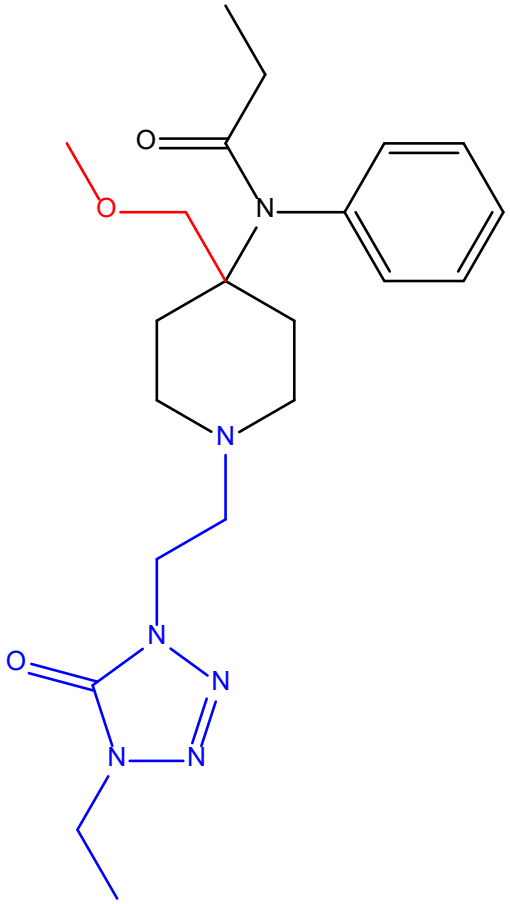


Carfentanil

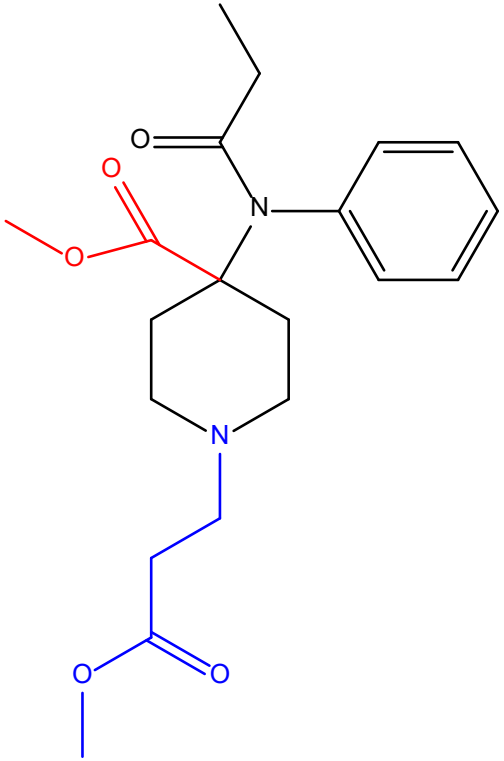


Four Phenethyl/Piperidine Substituted Fentalogs (R_2 & R_4)

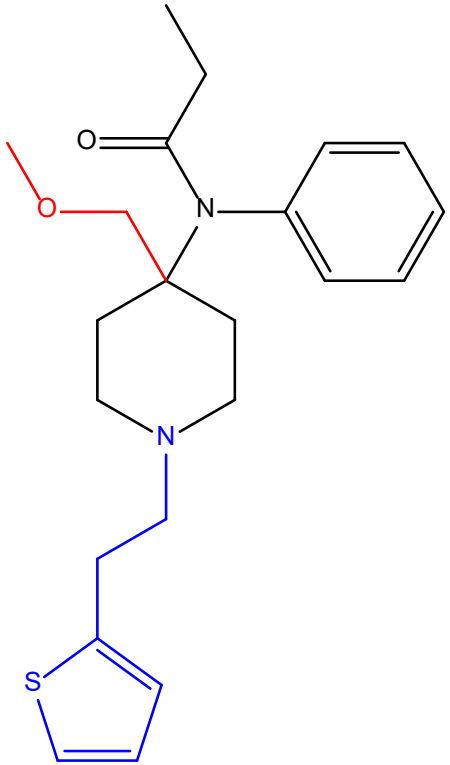
Alfentanil



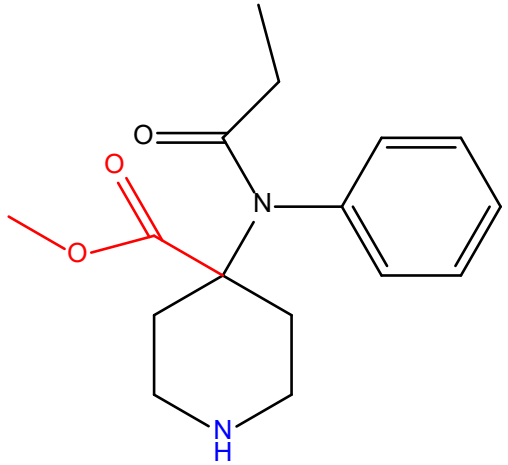
Remifentanil



Sufentanil



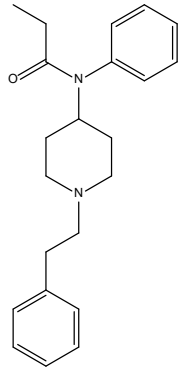
Norcarfentanil

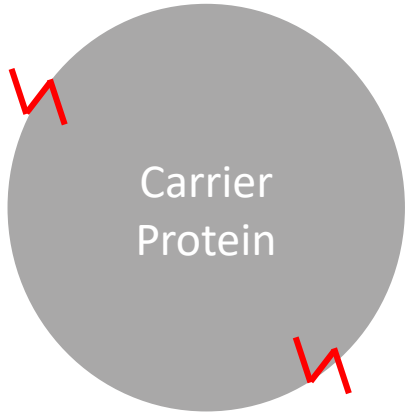


Background

- Enzyme Linked Immunosorbent Assay (ELISA) is the most commonly utilized immunoassay screening technique in forensic toxicology

- Drugs are too small to elicit an immune response
  - Bioconjugation with larger carrier protein





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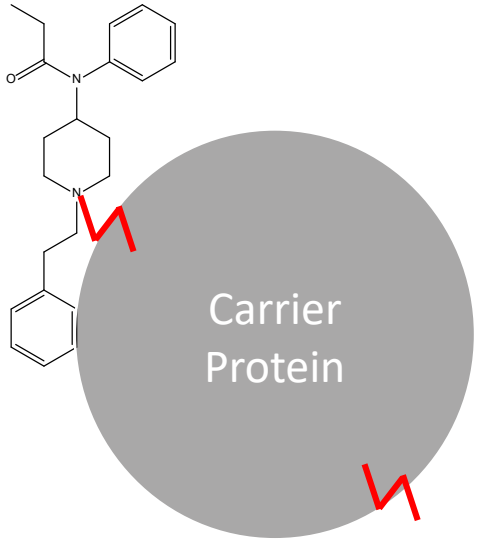

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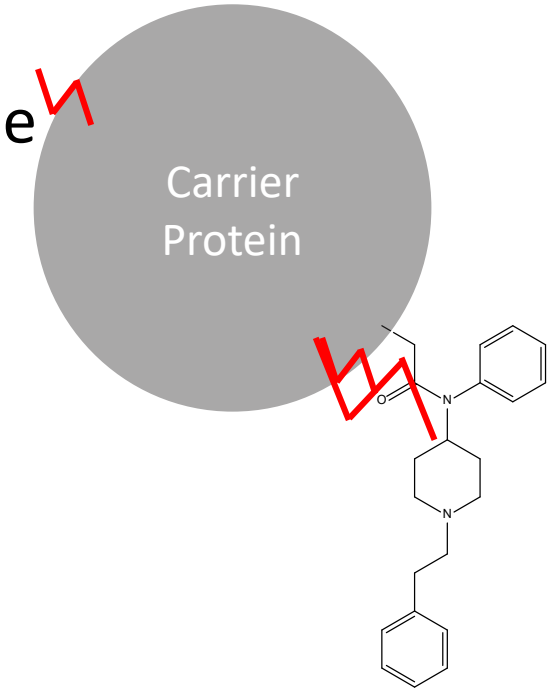


Background

- Enzyme Linked Immunosorbent Assay (ELISA) is the most commonly utilized immunoassay screening technique in forensic toxicology
- Drugs are too small to elicit an immune response
  - Bioconjugation with larger carrier protein
- Antibodies with high specificity reduce false positive and can be highly desirable (e.g. methamphetamine)
- However, poor specificity can be exploited for general screening purposes, allowing many drugs within a class to be identified (e.g. benzodiazepines)

Experimental Design

- Five commercial ELISA kits from three manufacturers
  - Randox Fentanyl
  - Randox Carfentanil/Remifentanil
  - Neogen Fentanyl Group
  - Neogen Fentanil Group
  - Immunalysis Fentanyl

- Instrumentation
  - Microtiter plate washer – BioTek ELX50/8
  - Microtiter plate reader – Dynex Technologies Opsys MR
    - 450 nm & 630 nm reference

- All assays performed in accordance with manufacturer recommendations in urine
- Dose-response curves used to calculate % cross-reactivity towards each compound

Experimental Conditions

Manufacturer	Target	Sample Volume	Conjugate Volume /Incubation time	TMB volume /incubation time	Stop Reagent Volume
Randox	Norfentanyl	50 µL[a]	75 µL/1 h	125 µL/20 min	100 µL
	Remifentanil	50 µL[a]	100 µL/1 h	125 µL/20 min	100 µL
Neogen	Fentanyl	20 µL	100 µL/45 min	100 µL/30 min	100 µL
	Alfentanil	20 µL	180 µL/45 min	120 µL/30 min	50 µL
Immunalysis	Fentanyl	20 µL	100 µL/1 h	100 µL/30 min	100 µL

[a]Diluted 1:4 with wash buffer prior to sample addition

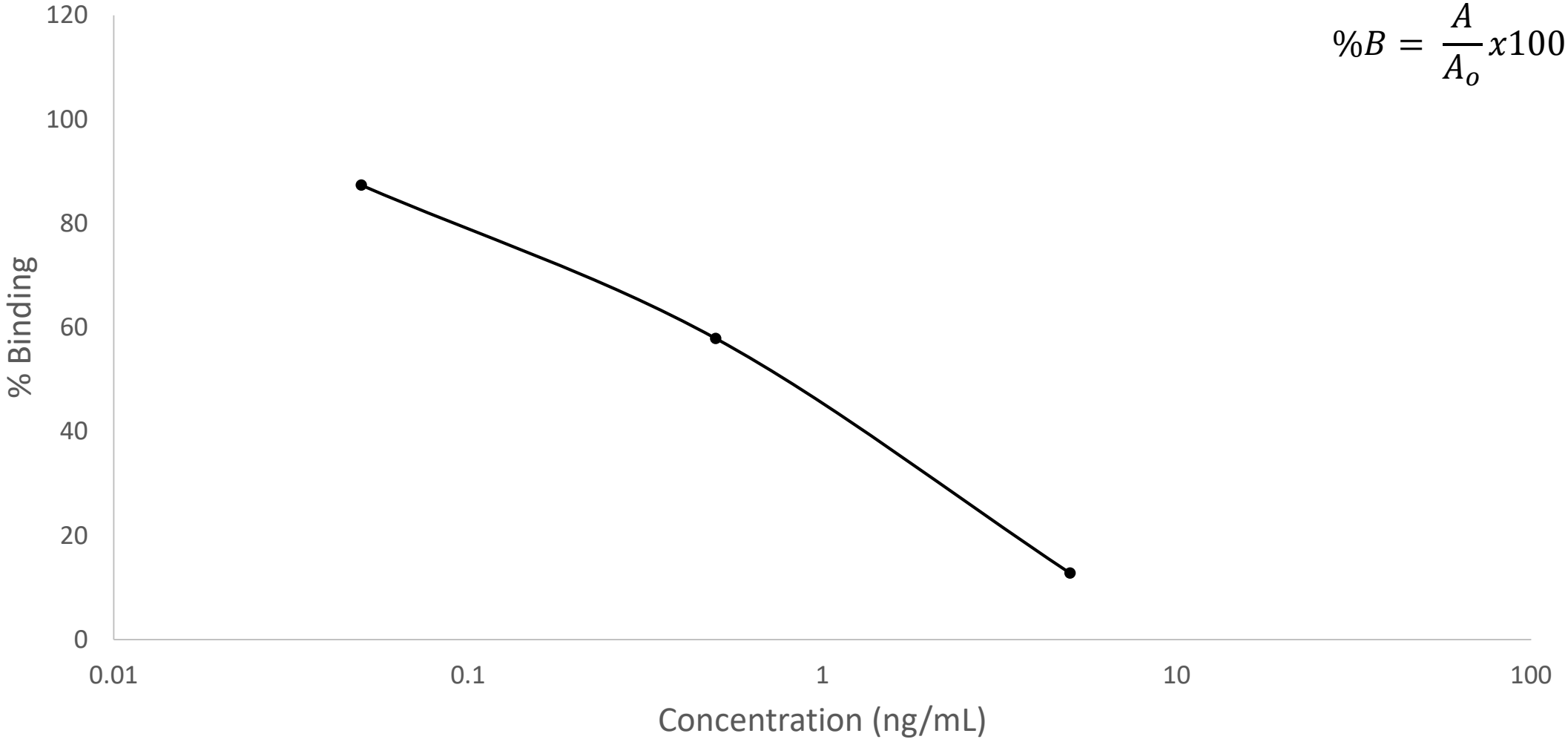
Cross-reactivity

- Variable response of an antibody to an antigen

- 50% CR = **double** concentration to produce response equivalent to target analyte
- 100% CR = **same** concentration to produce response equivalent to target analyte
- 200% CR = **half** concentration to produce response equivalent to target analyte

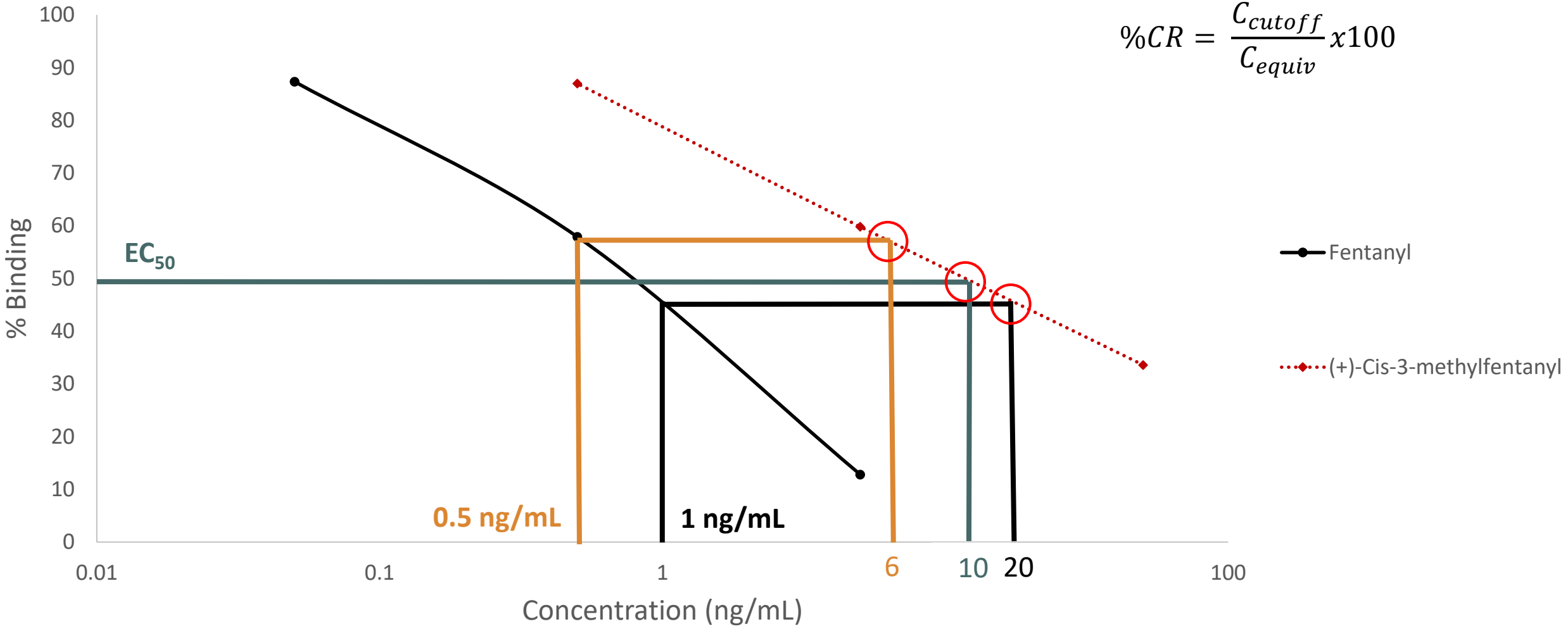
Dose-Response Curve

$$\%B = \frac{A}{A_o} x100$$

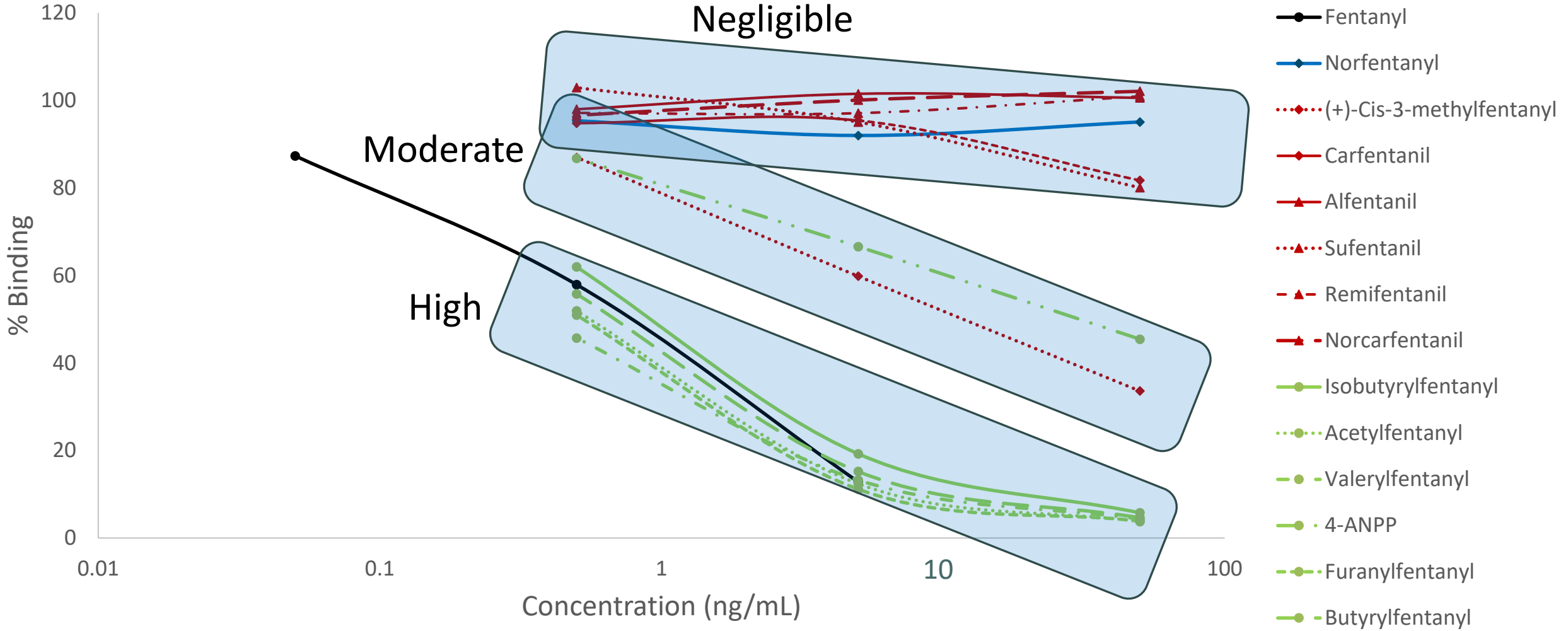


Dose-Response Curve

$$\%CR = \frac{C_{cutoff}}{C_{equiv}} x100$$



Dose-Response Curve



Randox Fentanyl ELISA

			% Cross-Reactivity		
Analog	Modification	Manufacturer	EC50	0.5 ng/ml equiv	1 ng/ml equiv
Norfentanyl	Phenethyl	100	100	100	100
Fentanyl	-	790	720	250	333
(+)-Cis-3-methylfentanyl	Piperidine	31	<5	50	25
Carfentanil	Piperidine	<1	<1	<1	<2
Alfentanil	Piperidine, phenethyl	<1	<1	<1	<2
Sufentanil	Piperidine, phenethyl	<1	<1	<1	<2
Remifentanil	Piperidine, phenethyl	<1	<5	<5	<10
Norcarfentanil	Piperidine, phenethyl	-	<5	<5	<10
Isobutyrylfentanyl	*N*-acyl	-	144	143	119
Acetylfentanyl	*N*-acyl	37	<5	50	33
Valerylfentanyl	*N*-acyl	-	60	67	67
4-ANPP	*N*-acyl	<1	<1	<1	<2
Furanylfentanyl	*N*-acyl	-	400	357	333
Butyrylfentanyl	*N*-acyl	-	450	250	294

Randox Carfentanil/Remifentanil ELISA

			% Cross-Reactivity		
Analog	Modification	Manufacturer	EC50	0.5 ng/ml equiv	1 ng/ml equiv
Norfentanyl	Phenethyl	-	<1	<1	<2
Fentanyl	-	<1	<1	<1	<2
(+)-Cis-3-methylfentanyl	Piperidine	<1	<1	<1	<2
Carfentanil	Piperidine	162	115	111	91
Alfentanil	Piperidine, phenethyl	30	38	38	<20
Sufentanil	Piperidine, phenethyl	13	15	<10	<20
Remifentanil	Piperidine, phenethyl	100	100	100	100
Norcarfentanil	Piperidine, phenethyl	<5	91	91	71
Isobutyrylfentanyl	*N*-acyl	-	<1	<1	<2
Acetylfentanyl	*N*-acyl	-	<1	<1	<2
Valerylfentanyl	*N*-acyl	-	<1	<1	<2
4-ANPP	*N*-acyl	-	<1	<1	<2
Furanylfentanyl	*N*-acyl	-	<1	<1	<2
Butyrylfentanyl	*N*-acyl	-	<1	<1	<2

Neogen Fentanyl Group Kit

			% Cross-Reactivity		
Analog	Modification	Manufacturer	EC50	0.5 ng/ml equiv	1 ng/ml equiv
Norfentanyl	Phenethyl	<1	<1	<1	<2
Fentanyl	-	100	100	100	100
(+)-Cis-3-methylfentanyl	Piperidine	50	3	13	4
Carfentanil	Piperidine	6	5	25	6
Alfentanil	Piperidine, phenethyl	<1	<1	<1	<2
Sufentanil	Piperidine, phenethyl	<1	<1	<1	<2
Remifentanil	Piperidine, phenethyl	<1	<1	<1	<2
Norcarfentanil	Piperidine, phenethyl	-	<1	<1	<2
Isobutyrylfentanyl	*N*-acyl	66	104	102	101
Acetylfentanyl	*N*-acyl	42	87	83	83
Valerylfentanyl	*N*-acyl	208	81	71	80
4-ANPP	*N*-acyl	<1	<1	2	<2
Furanylfentanyl	*N*-acyl	184	100	98	100
Butyrylfentanyl	*N*-acyl	<1	<5	<5	<10

Neogen Fentanil Group Kit

			% Cross-Reactivity		
Analog	Modification	Manufacturer	EC50	0.5 ng/ml equiv	1 ng/ml equiv
Norfentanyl	Phenethyl	<1	<1	<1	<2
Fentanyl	-	<5	<5	<5	<10
(+)-Cis-3-methylfentanyl	Piperidine	<1	<1	<1	<2
Carfentanil	Piperidine	88	324	>1600	435
Alfentanil	Piperidine, phenethyl	100	100	100	100
Sufentanil	Piperidine, phenethyl	270	524	>1600	625
Remifentanil	Piperidine, phenethyl	76	110	200	185
Norcarfentanil	Piperidine, phenethyl	-	17	15	19
Isobutyrylfentanyl	*N*-acyl	<1	<1	<1	<2
Acetylfentanyl	*N*-acyl	<1	<1	<1	<2
Valerylfentanyl	*N*-acyl	<1	<1	<1	<2
4-ANPP	*N*-acyl	<1	<1	<1	<2
Furanylfentanyl	*N*-acyl	<1	<1	<1	<2
Butyrylfentanyl	*N*-acyl	<1	<5	<5	<10

Immunalysis ELISA Plate

			% Cross-Reactivity		
Analog	Modification	Manufacturer	EC50	0.5 ng/ml equiv	1 ng/ml equiv
Norfentanyl	Phenethyl	<1	<1	<1	<2
Fentanyl	-	100	100	100	100
(+)-Cis-3-methylfentanyl	Piperidine	-	9	<5	<10
Carfentanil	Piperidine	-	<1	<1	<2
Alfentanil	Piperidine, phenethyl	-	<1	<1	<2
Sufentanil	Piperidine, phenethyl	-	<1	<1	<2
Remifentanil	Piperidine, phenethyl	-	<1	<1	<2
Norcarfentanil	Piperidine, phenethyl	-	<1	<1	<2
Isobutyrylfentanyl	*N*-acyl	-	88	68	53
Acetylfentanyl	*N*-acyl	-	277	161	111
Valerylfentanyl	*N*-acyl	-	124	114	71
4-ANPP	*N*-acyl	10	<5	<5	<10
Furanylfentanyl	*N*-acyl	-	212	156	111
Butyrylfentanyl	*N*-acyl	-	164	119	77

Discussion

- Immunalysis Fentanyl, Randox Fentanyl, and Neogen Fentanyl Group kits were effective detecting analogs modified at the *N*-acyl group (R_1)
  - 4-ANPP not highly cross-reactive with any of the tested kits
  - Randox Fentanyl only assay to detect both fentanyl and norfentanyl

Discussion

- Randox Carfentanil/Remifentanil and Neogen Fentanil Group kits effective were detecting analogs modified on both the piperidine ring (R_4) and at the phenethyl group (R_2)
  - Randox Carfentanil/Remifentanil highly specific for target similar compounds and carfentanil metabolite
- The Randox Fentanyl kit was the only kit able to detect (+)-Cis-3-methylfentanyl with marginal cross-reactivity
- **No kit was able to detect all modifications effectively**

Conclusions

- Bioconjugation is an inherent disadvantage of immunoassays
- Multiple kits required to effectively screen for broad range of modifications
- Purchasing multiple kits is cost prohibitive
- Alternative MS-based screening may be more responsive to the proliferation of NPSs

Forensic Toxicology
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SHORT COMMUNICATION

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Questions?

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