Supplementary Materials

Saline (vehicle control) or
0.03-1 mg/kg fentanyl
or 1-30 mg/kg morphine, sc.

Saline (naloxone control)
sc

| Baseline Phase: 20 min
No injections; recording
mice's acclimation to
chamber | 5 groups (saline + 4 doses of
MOR agonist)/session, n =
8/group

Injections → | Agonist Phase: 35 Min
Recording begins
immediately after sc
injections with saline or
MOR agonist | All groups

Injections → |

Reversal Phase: 35 Min
Recording begins
immediately after sc
saline injections

Total Recording Time: 90 min

Parameters Recorded: Minute
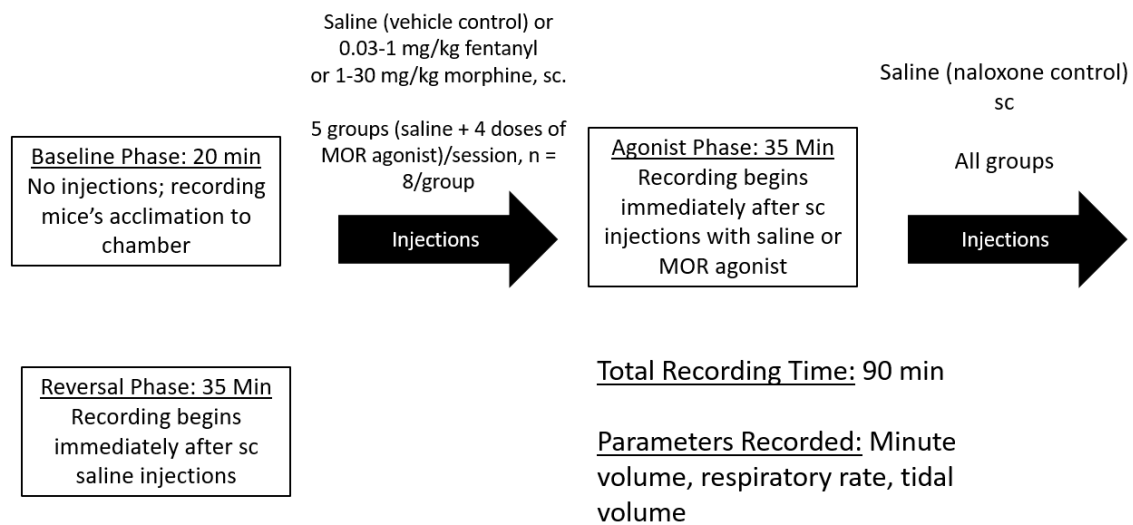volume, respiratory rate, tidal
volume

Figure S1. Diagram of Three-Phase Whole-Body Plethysmography Protocol
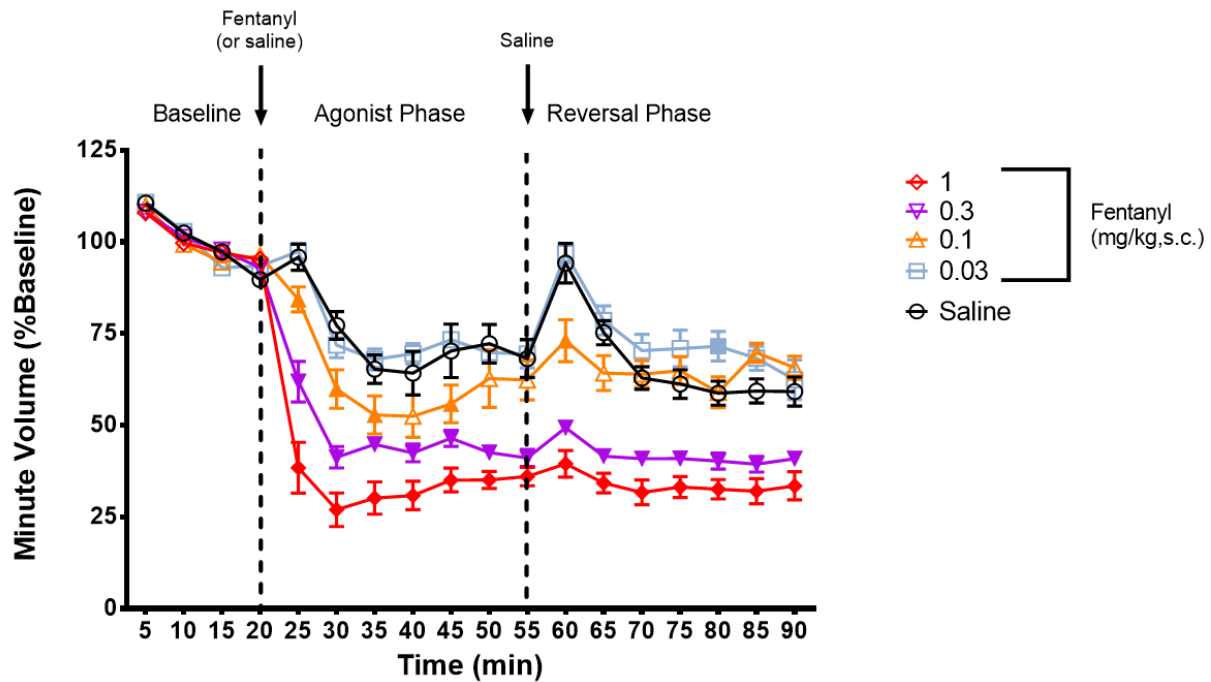MOR agonist: μ-opioid receptor agonist

Figure S2. Fentanyl Demonstrates Dose Response Effects in Induction of Respiratory Depression (Specifically, MVb) in Mice, with Sustained Respiratory Depression at 0.3 mg/kg and Higher.

After the first 20 min (Baseline Phase), during which baseline respiration was established, male Swiss Webster mice were injected with either vehicle control (saline) or 0.03-1.0 mg/kg fentanyl sc and MVb (minute volume; product of frequency and tidal volume), measured for 35 min (Agonist Phase). Mice then received a saline injection, and MVb was measured for an additional 35 min (Reversal Phase). 2-way repeated-measures ANOVA revealed a significant effect of dose ($P<0.0001$) and time ($P<0.0001$), with differences from control further evaluated by Holm-Šídák post hoc. Filled symbols denote significant difference from saline ($p \leq 0.05$). Data are presented as mean normalized MVb (5-min bins), expressed as percent baseline ± SEM. n = 8/group
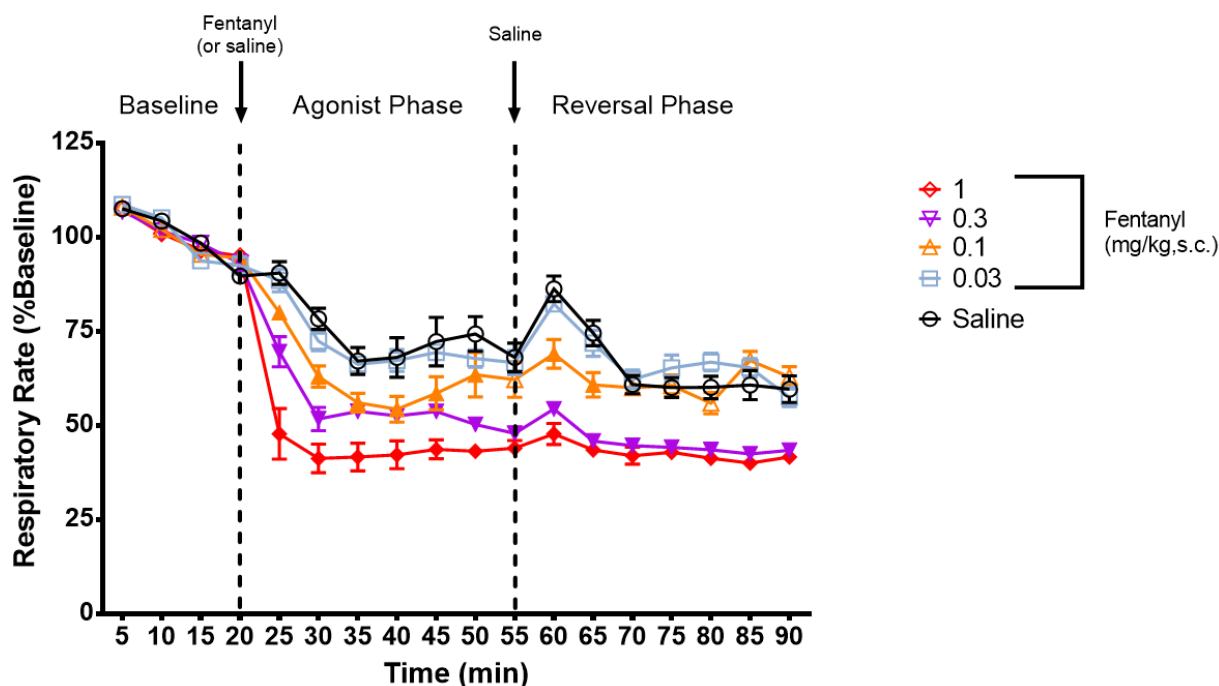
Figure S3. Fentanyl Demonstrates Dose Response Effects in Induction of Respiratory Depression (Specifically, f) in Mice, with Sustained Respiratory Depression at 0.3 mg/kg and Higher.

After the first 20 min (Baseline Phase), during which baseline respiration was established, male Swiss Webster mice were injected with either vehicle control (saline) or 0.03-1.0 mg/kg fentanyl sc and f (frequency, or respiratory rate; breaths/minute) measured for 35 min (Agonist Phase). Mice then received a saline injection, and f was measured for an additional 35 min (Reversal Phase). 2-way repeated-measures ANOVA revealed a significant effect of dose (P<0.0001) and time (P<0.0001), with differences from control further evaluated by Holm-Šídák post hoc. Filled symbols denote significant difference from saline (p≤0.05). Data are presented as mean normalized f (5-min bins), expressed as percent baseline ± SEM. n = 8/group
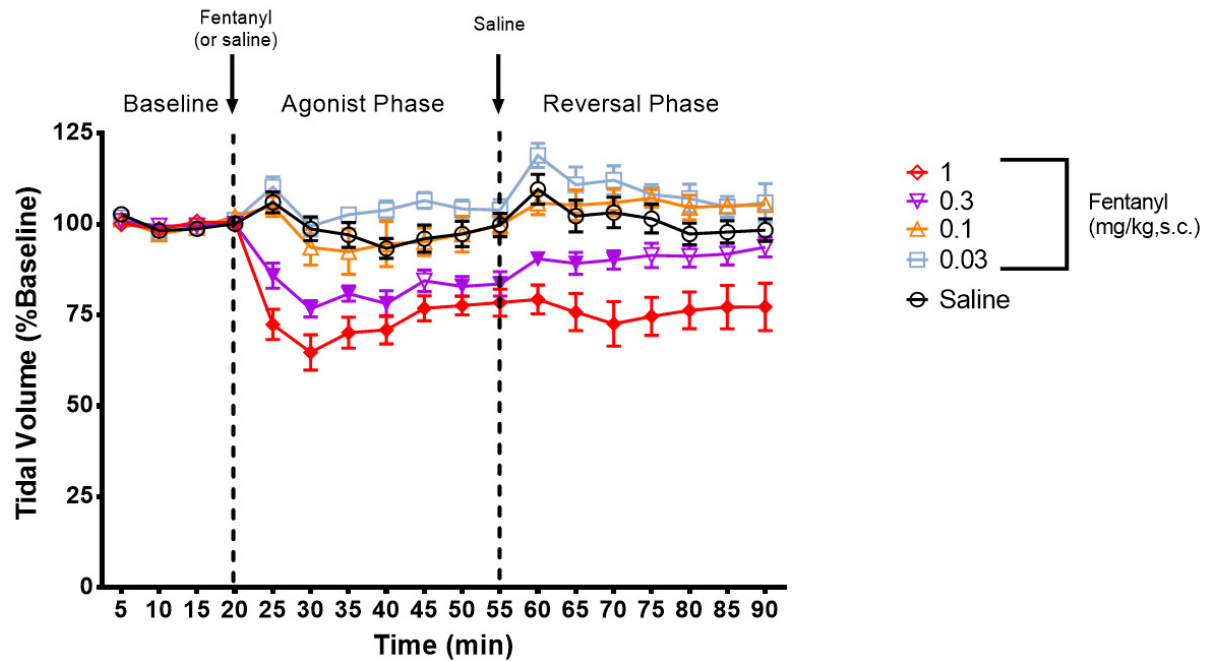
Figure S4. Fentanyl Demonstrates Dose Response Effects in Induction of Respiratory Depression (Specifically, TVb) in Mice, with Sustained Respiratory Depression at 0.3 mg/kg and Higher.

After the first 20 min (Baseline Phase), during which baseline respiration was established, male Swiss Webster mice were injected with either vehicle control (saline) or 0.03-1.0 mg/kg fentanyl sc and TVb (tidal volume; volume of air displaced from the lungs/respiratory cycle) measured for 35 min (Agonist Phase). Mice then received a saline injection, and TVb was measured for an additional 35 min (Reversal Phase). 2-way repeated-measures ANOVA revealed a significant effect of dose ($P<0.0001$) and time ($P<0.0001$), with differences from control further evaluated by Holm-Šídák post hoc. Filled symbols denote significant difference from saline ($p≤0.05$). Data are presented as mean normalized TVb (5-min bins), expressed as percent baseline ± SEM. n = 8/group
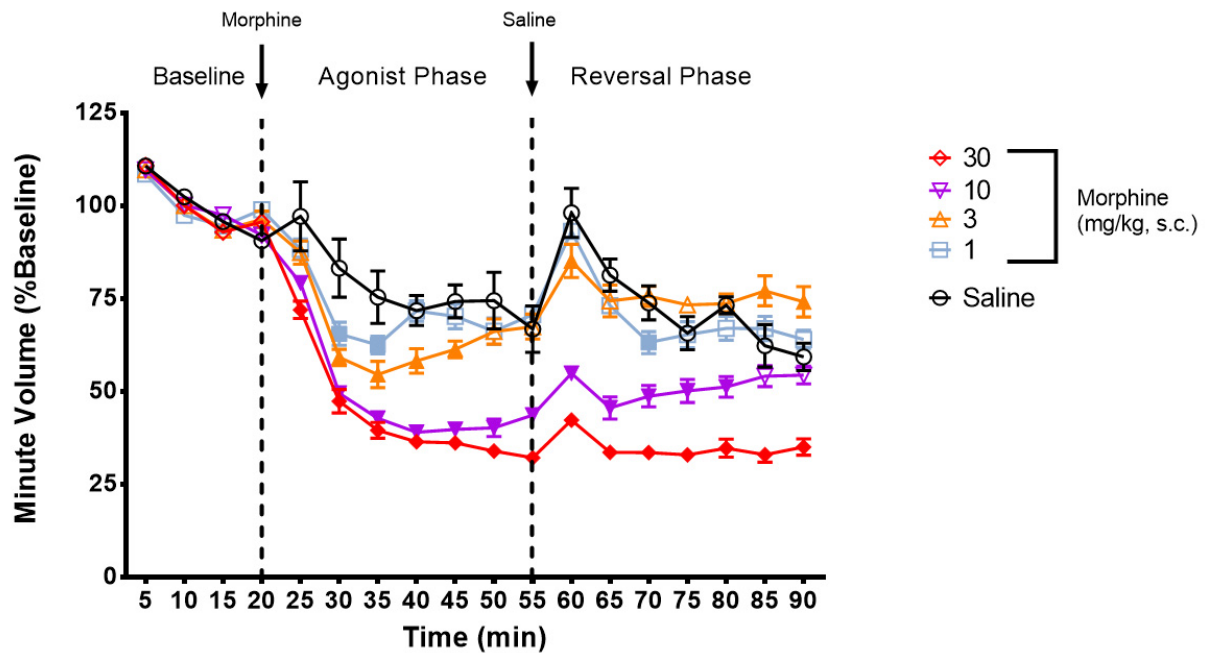
Figure S5. Morphine Demonstrates Dose Response Effects in Induction of Respiratory Depression (Specifically, MVb) in Mice, with Sustained Respiratory Depression at 30 mg/kg.

After the first 20 min (Baseline Phase), during which baseline respiration was established, male Swiss Webster mice were injected with either vehicle control (saline) or 1-30 mg/kg morphine sc and MVb (minute volume; product of frequency and tidal volume) measured for 35 min (Agonist Phase). Mice then received a saline injection, and MVb was measured for an additional 35 min (Reversal Phase). 2-way repeated-measures ANOVA revealed a significant effect of dose ($P<0.0001$) and time ($P<0.0001$), with differences from control further evaluated by Holm-Šídák post hoc. Filled symbols denote significant difference from saline ($p≤0.05$). Data are presented as normalized mean MVb (5-min bins), expressed as percent baseline ± SEM. n = 8/group
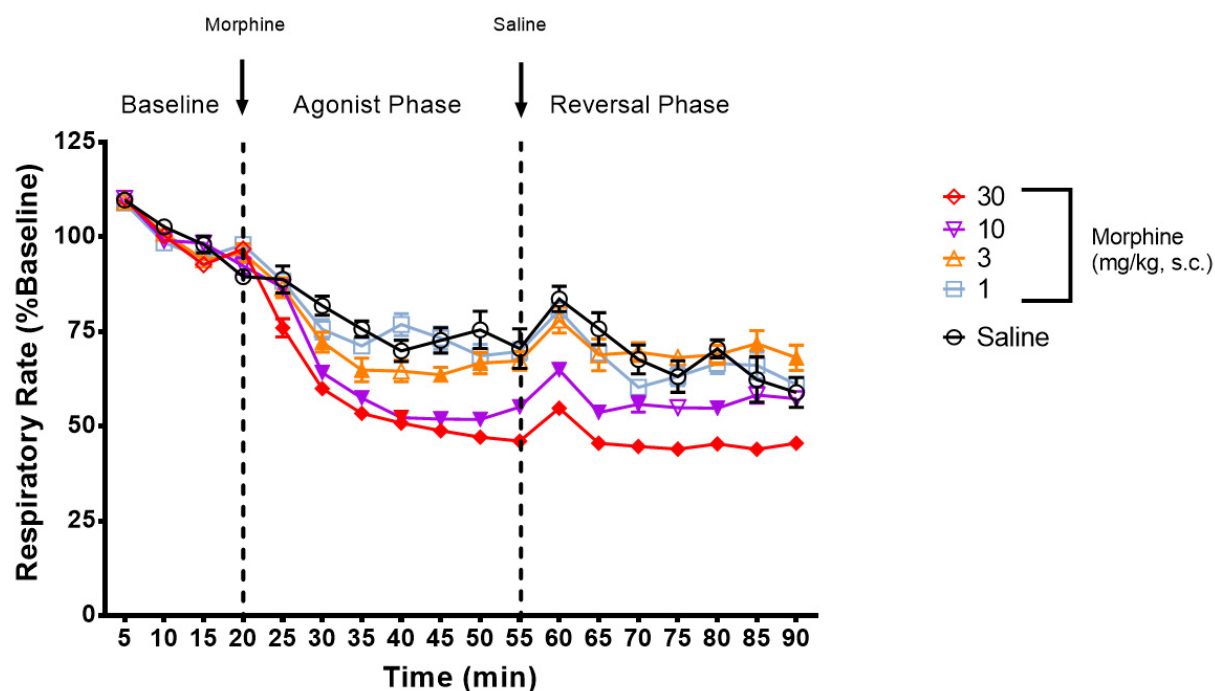
Figure S6. Morphine Demonstrates Dose Response Effects in Induction of Respiratory Depression (Specifically, f) in Mice, with Sustained Respiratory Depression at 30 mg/kg.

After the first 20 min (Baseline Phase), during which baseline respiration was established, male Swiss Webster mice were injected with either vehicle control (saline) or 1-30 mg/kg morphine sc and f (frequency, or respiratory rate; breaths/minute) measured for 35 min (Agonist Phase). Mice then received a saline injection, and f was measured for an additional 35 min (Reversal Phase). 2-way repeated-measures ANOVA revealed a significant effect of dose (P<0.0001) and time (P<0.0001), with differences from control further evaluated by Holm-Šídák post hoc. Filled symbols denote significant difference from saline (p≤0.05). Data are presented as normalized f (5-min bins), expressed as percent baseline ± SEM. n = 8/group
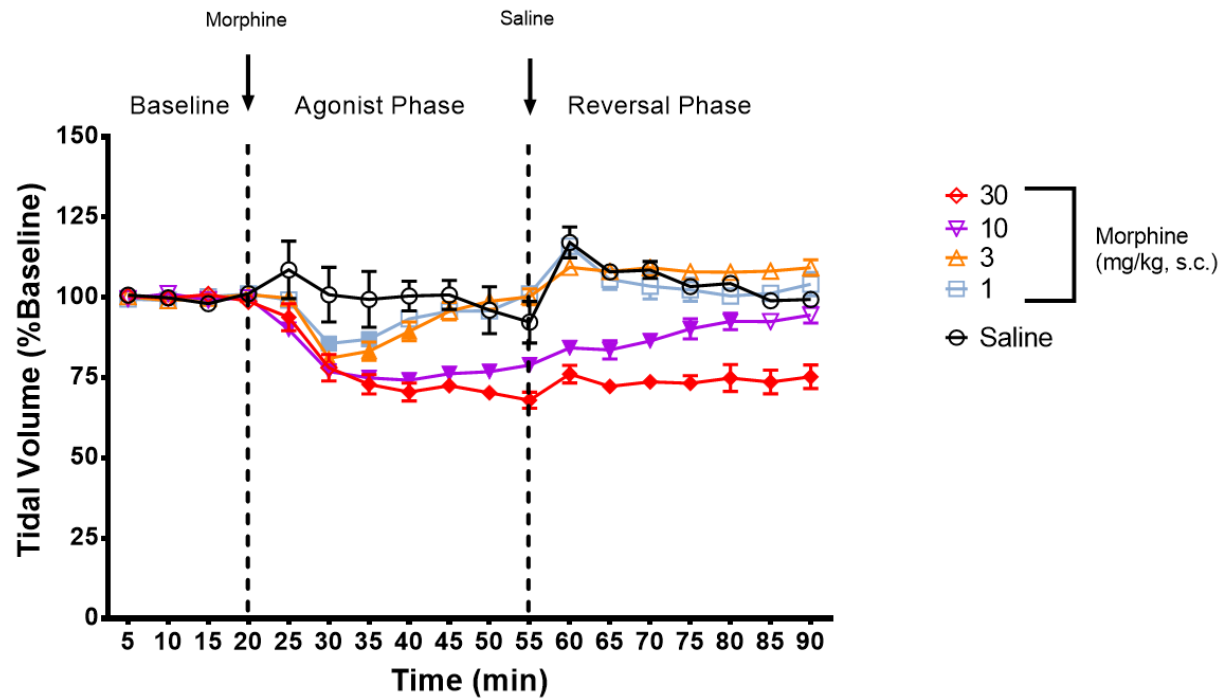
Figure S7. Morphine Demonstrates Dose Response Effects in Induction of Respiratory Depression (Specifically, TVb) in Mice, with Sustained Respiratory Depression at 30 mg/kg.

After the first 20 min (Baseline Phase), during which baseline respiration was established, male Swiss Webster mice were injected with either vehicle control (saline) or 1-30 mg/kg morphine sc and TVb (tidal volume; volume of air displaced from the lungs/respiratory cycle) measured for 35 min (Agonist Phase). Mice then received a saline injection, and TVb was measured for an additional 35 min (Reversal Phase). 2-way repeated-measures ANOVA revealed a significant effect of dose (P<0.0001) and time (P<0.0001), with differences from control further evaluated by Holm-Šídák post hoc. Filled symbols denote significant difference from saline (p≤0.05). Data are presented as normalized mean TVb (5-min bins), expressed as percent baseline ± SEM. n = 8/group

Figure S8. Comparison of Respiratory Depressant Effects (Specifically, MVb) of 0.3 mg/kg Fentanyl sc and 30 mg/kg Morphine sc in Mice.

Data taken from Figures 4.4 and 4.7, respectively. All phases as described in previous figure legends. 2-way repeated-measures ANOVA with Holm-Šídák post hoc as needed. Filled symbols denote significant difference between groups ($p \leq 0.05$). Data are presented as normalized mean MVb (5-min bins), expressed as percent baseline ± SEM. n = 8/group
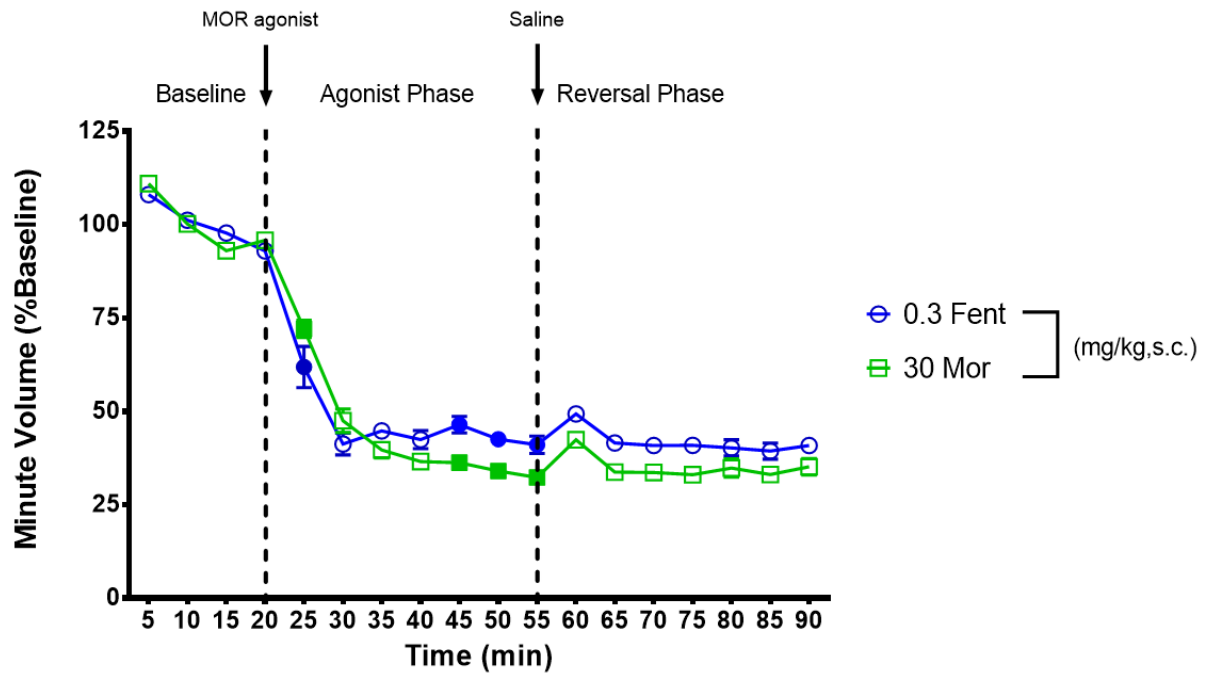
Figure S9. Comparison of Respiratory Depressant Effects (Specifically, f) of 0.3 mg/kg Fentanyl sc and 30 mg/kg Morphine sc in Mice.

Data taken from Figures 4.5 and 4.8, respectively. All phases as described in previous figure legends. 2-way repeated-measures ANOVA with Holm-Šídák post hoc as needed. Filled symbols denote significant difference between groups ($p \leq 0.05$). Data are presented as normalized mean f (5-min bins), expressed as percent baseline ± SEM. n = 8/group
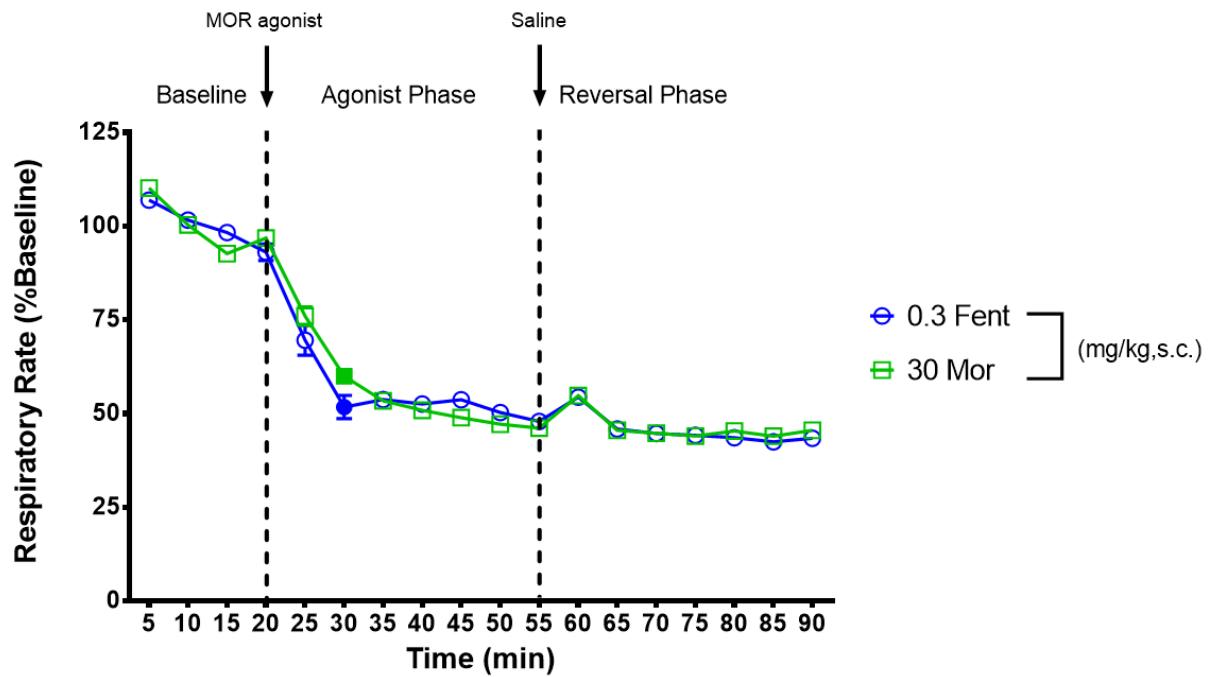
Figure S10. Comparison of Respiratory Depressant Effects (Specifically, TVb) of 0.3 mg/kg Fentanyl sc and 30 mg/kg Morphine sc in Mice.

Data taken from Figures 3 and 6, respectively. All phases as described in previous figure legends. 2-way repeated-measures ANOVA with Holm-Šídák post hoc as needed. Filled symbols denote significant difference between groups (p≤0.05). Data are presented as normalized mean TVb (5-min bins), expressed as percent baseline ± SEM. n = 8/group

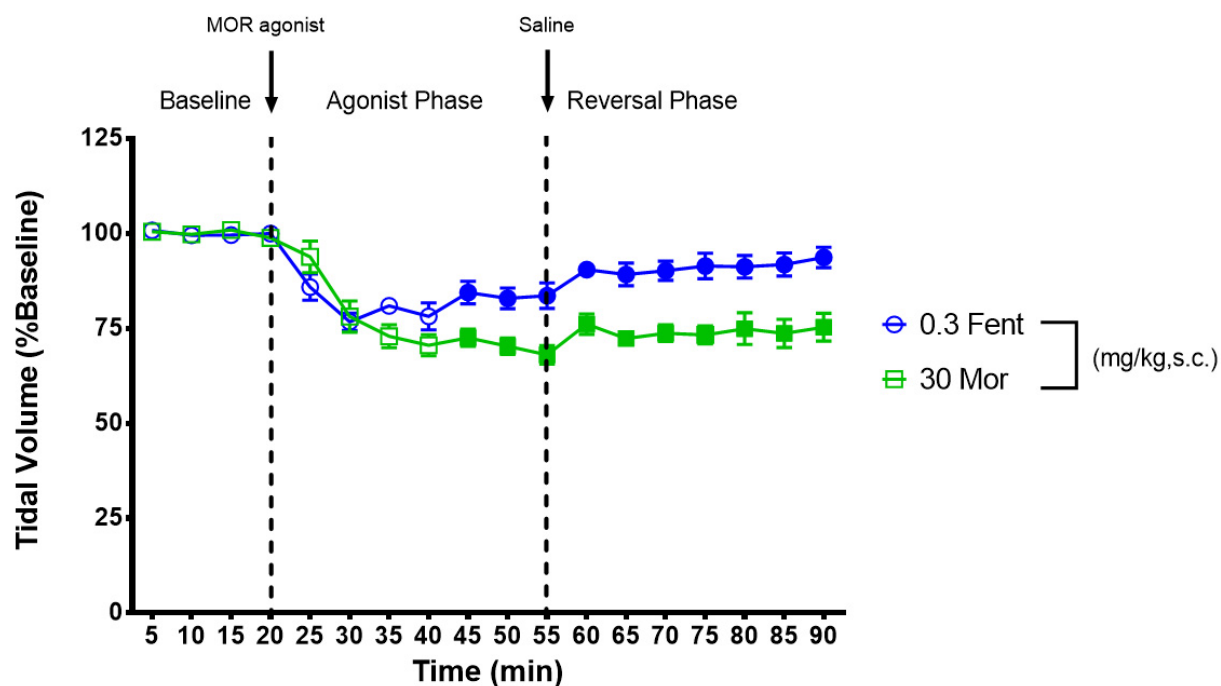**Bioanalytical Method Validation**

Standards and reagents were as described in **Section 2.2**. Solid-phase extraction and liquid chromatography-tandem mass spectrometry were performed as described in **Section 2.6 and 2.7**, respectively.

Samples for most method validation procedures were obtained from sterile-filtered, heat-inactivated mouse serum stock (SKU: 30611146-3; Lot No. V20082500) purchased from bioWORLD (Dublin, OH, USA). Samples for quality controls (brain, lung, etc.) were harvested from drug-naïve male Swiss Webster mice that could be used as sources of blank tissue. Mice were decapitated by guillotine, and trunk blood and tissue samples (brain, liver, lung, heart, kidney, spleen, small intestine, large intestine, stomach, muscle, fat, and skin) were immediately harvested on ice. After collection, samples were stored at –80˚C.

Table S1. Parent Ion (Q1) and Product Ion (Q3) Mass-to-Charge Ratios, MRM Transitions, Collision Energy (CE) and Declustering Potential (DP) for Spectrometric Analysis

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	CE (eV)	DP (V)
Fentanyl (Quant.)	337	188	30	62
Fentanyl (Qual.)	337	105	42	62
Fentanyl-d5 (Quant.)	342	188	30	62
Fentanyl-d5 (Qual.)	342	105	42	62
Norfentanyl (Quant.)	233	84	22	67
Norfentanyl (Qual.)	233	150	24	67
Norfentanyl-d5 (Quant.)	238	84	22	67
Norfentanyl-d5 (Qual.)	238	155	24	67
4-ANPP (Quant.)	281	188	22	63
4-ANPP (Qual.)	281	105	38	63
4-ANPP-d5 (Quant.)	286	188	22	63
4-ANPP-d5 (Qual.)	286	105	38	63
Morphine (Quant.)	286	185	41	127
Morphine (Qual.)	286	157	54	127
Morphine-d3 (Quant.)	289	185	45	127
Morphine-d3 (Qual.)	289	157	54	127
Morphine-3-β-D-glucuronide (Quant.)	462	286	40	30
Morphine-3-β-D-glucuronide (Qual.)	462	201	56	30
Morphine-3-β-D-glucuronide (Qual.)	462	165	71	30
Morphine-3-β-D-glucuronide-d3 (Quant.)	465	289	40	30
Morphine-3-β-D-glucuronide-d3 (Qual.)	465	204	56	30
Morphine-3-β-D-glucuronide-d3 (Qual.)	465	165	71	30

Q1: Quadrupole 1
Q3: Quadrupole 3
Quant.: Quantifying MRM (multiple reaction monitoring) transitions (Q1→Q3)
Qual.: Qualifying MRM transitions (Q1→Q3)
CE: Collision Energy; rate of acceleration at which ions collide with inert gas in Quadrupole 2
DP: Declustering Potential; applied voltage that prevents ion clustering

Table S2. Validation Summary Table for Fentanyl

Method Description	
Short description of method	Solid-phase extraction with reverse-phase HPLC with MS/MS detection
Matrix	Mouse serum
Analyte	Fentanyl
Internal standard (IS)	Fentanyl-d5 (deuterated Fentanyl)
Calibration concentrations	1 ng/mL to 100 ng/mL
QC concentrations	1 ng/mL (LLOQ), 3 ng/mL (Low QC), 7.5 ng/mL (Med QC), and 75 ng/mL (High QC)
Selectivity	No peaks
Lower limit of quantification	1 ng/mL Between-run absolute bias (accuracy): 11% Between-run precision: 15% Within-run absolute bias (accuracy): 2-16% Within-run precision: 4-22%
Between-run absolute bias (accuracy)*	4%-11%
Between-run precision	7%-16%
Within-run absolute bias (accuracy)*	Run 1: 3-14% Run 2: 8-15% Run 3: 2-11%
Within-run precision	Run 1: 4-16% Run 2: 3-22% Run 3: 4-14%
Matrix effect	Low QC: 45% (18% CV) High QC: 15% (7% CV)
Recovery of analyte	66%-93%
Recovery of IS	93%
Auto-sampler storage stability	Confirmed for up to 72 hr at 40°C nominal 24 hr: Bias (accuracy) 8% for LLOQ, 4% for Low QC, 7% for Med QC, and 8% for High QC 48 hr: Bias (accuracy) 7% for LLOQ, 7% for Low QC, 5% for Med QC, and 10% for High QC 72 hr: Bias (accuracy) 1% for LLOQ, 3% for Low QC, 10% for Med QC, and 2% for High QC
Freeze-thaw stability	Confirmed up to 3 cycles Bias (accuracy) 5% for Low QC and 12% for High QC
Bench top stability	Confirmed up to 24 hr at room temperature Bias (accuracy) 4% for Low QC and 9% for High QC
Injector carryover	No carryover was observed
Long-term storage stability	Confirmed up to 138 days Bias (accuracy) 13% for Low QC, 0.1% for Med QC, and 18% for High QC

* Low bias = high accuracy

Table S3. Validation Summary Table for Norfentanyl

Method Description	
Short description of method	Solid-phase extraction with reverse-phase HPLC with MS/MS detection
Matrix	Mouse serum
Analyte	Norfentanyl
Internal standard (IS)	Norfentanyl-d5 (deuterated Norfentanyl)
Calibration concentrations	5 ng/mL to 500 ng/mL
QC concentrations	5 ng/mL (LLOQ), 15 ng/mL (Low QC), 40 ng/mL (Med QC), and 400 ng/mL (High QC)
Selectivity	No peaks
Lower limit of quantification	5 ng/mL Between-run absolute bias (accuracy): 4% Between-run precision: 11% Within-run absolute bias (accuracy): 3-16% Within-run precision: 2-7%
Between-run absolute bias (accuracy)	0.5%-4%
Between-run precision	9%-15%
Within-run absolute bias (accuracy)	Run 1: 4-16% Run 2: 3-13% Run 3: 3-9%
Within-run precision	Run 1: 6-18% Run 2: 5-7% Run 3: 3-7%
Matrix effect	Low QC: 15% (13% CV) High QC: 6% (14% CV)
Recovery of analyte	101-139%
Recovery of IS	118%
Auto-sampler storage stability	Confirmed for up to 72 hr at 40°C nominal 24 hr: Bias (accuracy) 11% for LLOQ, 6% for Low QC, 2% for Med QC, and 9% for High QC 48 hr: Bias (accuracy) 7% for LLOQ, 6% for Low QC, 3% for Med QC, and 8% for High QC 72 hr: Bias (accuracy) 6% for LLOQ, 15% for Low QC, 5% for Med QC, and 5% for High QC
Freeze-thaw stability	Confirmed up to 3 cycles Bias (accuracy) 10% for Low QC and 5% for High QC
Bench top stability	Confirmed up to 24 hr at room temperature
Injector carryover	No carryover was observed
Long-term storage stability	Confirmed up to 138 days Bias (accuracy) 15% at Low QC, 2% at Med QC, and 13% at High QC

* Low bias = high accuracy

Table S4. Validation Summary Table for 4-ANPP

Method Description	
Short description of method	Solid-phase extraction with reverse-phase HPLC with MS/MS detection
Matrix	Mouse serum
Analyte	N-Phenyl-1-(2-phenethyl)-4-piperidinamine
Internal standard (IS)	4-ANPP-d5 (deuterated 4-ANPP)
Calibration concentrations	1 ng/mL to 100 ng/mL
QC concentrations	1 ng/mL (LLOQ), 3 ng/mL (Low QC), 7.5 ng/mL (Med QC), and 75 ng/mL (High QC)
Selectivity	No peaks
Lower limit of quantification	1 ng/mL Between-run absolute bias (accuracy): 8% Between-run precision: 10% Within-run absolute bias (accuracy): 1-16% Within-run precision: 6-9%
Between-run absolute bias (accuracy)*	6-14%
Between-run precision	6-13%
Within-run absolute bias (accuracy)*	Run 1: 3-8% Run 2: 15-20% Run 3: 1-18%
Within-run precision	Run 1: 4-14% Run 2: 1-7% Run 3: 6-9%
Matrix effect	Low QC: 90% (9% CV) High QC: 14% (5% CV)
Recovery of analyte	69%-88%
Recovery of IS	85%
Auto-sampler storage stability	Confirmed for up to 72 hr at 40°C nominal 24 hr: Bias (accuracy) 1% for LLOQ, 4% for Low QC, 7% for Med QC, and 11% for High QC 48 hr: Bias (accuracy) 10% for LLOQ, 5% for Low QC, 5% for Med QC, and 19% for High QC 72 hr: Bias (accuracy) 4% for LLOQ, 8% for Low QC, 18% for Med QC, and 13% for High QC
Freeze-thaw stability	Confirmed up to 3 cycles Bias (accuracy) 3% for Low QC and 8% for High QC
Bench top stability	Confirmed up to 24 hr at room temperature Bias (accuracy) 8% for Low QC and 12% for High QC
Injector carryover	No carryover was observed**
Long term storage stability	Confirmed up to 138 days Bias (accuracy) 18% for Low QC, 0.3% for Med QC, and 19% for High QC

* Low bias = high accuracy
** Single exception: 50 ng/mL measured in double blank during Run 1

Table S5. Validation Summary Table for Morphine

Method Description	
Short description of method	Solid-phase extraction with reverse-phase HPLC with MS/MS detection
Matrix	Mouse serum
Analyte	Morphine
Internal standard (IS)	Morphine-d3 (deuterated Morphine)
Calibration concentrations	10 ng/mL to 1000 ng/mL
QC concentrations	10 ng/mL (LLOQ), 30 ng/mL (Low QC), 75 ng/mL (Med QC), and 750 ng/mL (High QC)
Selectivity	No peaks
Lower limit of quantification	10 ng/mL Between-run absolute bias (accuracy): 4% Between-run precision: 13% Within-run absolute bias (accuracy): 3-10% Within-run precision: 5-19%
Between-run absolute bias (accuracy)*	1-10%
Between-run precision	5-13%
Within-run absolute bias (accuracy)*	Run 1: 1-11% Run 2: 10-15% Run 3: 5-10%
Within-run precision	Run 1: 5-18% Run 2: 2-8% Run 3: 5-7%
Matrix effect	Low QC: 19% (13% CV) High QC: 11% (1% CV)
Recovery of analyte	77-90%
Recovery of IS	93%
Auto-sampler storage stability	Confirmed for up to 72 hr at 40°C nominal 24 hr: Bias (accuracy) 1% for LLOQ, 7% for Low QC, 14% for Med QC, and 12% for High QC 48 hr: Bias (accuracy) 8% for LLOQ, 8% for Low QC, 12% for Med QC, and 8% for High QC 72 hr: Bias (accuracy) 1% for LLOQ, 10% for Low QC, 12% for Med QC, and 10% for High QC
Freeze-thaw stability	Confirmed up to 3 cycles Bias (accuracy) 12% for Low QC and 11% for High QC
Bench top stability	Confirmed up to 24 hr at room temperature Bias (accuracy) 9% for Low QC and 11% for High QC
Injector carryover	No carryover was observed
Long term storage stability	Confirmed up to 138 days Bias (accuracy) 9% for Low QC, 16% for Med QC, and 15% for High QC

* Low bias = high accuracy

Table S6. Validation Summary Table for Morphine-3-β-D-Glucuronide

Method Description	
Short description of method	Solid-phase extraction with reverse-phase HPLC with MS/MS detection
Matrix	Mouse serum
Analyte	Morphine-3-β-D-Glucuronide
Internal standard (IS)	Morphine-3-β-D-Glucuronide-d3 (deuterated Morphine-3-β-D-Glucuronide)
Calibration concentrations	50 ng/mL to 5000 ng/mL
QC concentrations	50 ng/mL (LLOQ), 150 ng/mL (Low QC), 400 ng/mL (Med QC), and 4000 ng/mL (High QC)
Selectivity	No peaks
Lower limit of quantification	50 ng/mL Between-run absolute bias (accuracy): 12% Between-run precision: 15% Within-run absolute bias (accuracy): 5-30% Within-run precision: 5-8%
Between-run absolute bias (accuracy)*	4-12%
Between-run precision	7-15%
Within-run absolute bias (accuracy)*	Run 1: 2-9% Run 2: 9-19% Run 3: 0.1-6%
Within-run precision	Run 1: 4-10% Run 2: 1-8% Run 3: 5-12%
Matrix effect	Low QC: 26% (16% CV) High QC: 12% (3% CV)
Recovery of analyte	66-81%
Recovery of IS	84%
Auto-sampler storage stability	Confirmed for up to 72 hr at 40°C nominal 24 hr: Bias (accuracy) 4% at LLOQ, 0.3% at Low QC, 3% at Med QC, and 3% at High QC 48 hr: Bias (accuracy) 1% at LLOQ, 6% at Low QC, 9% at Med QC, and 1% at High QC 72 hr: Bias (accuracy) 10% at LLOQ, 18% at Low QC, 12% at Med QC, and 7% at High QC
Freeze-thaw stability	Confirmed up to 3 cycles Bias (accuracy) 16% for Low QC and 17% for High QC
Bench top stability	Confirmed up to 24 hr at room temperature Bias (accuracy) 6% for Low QC and 16% for High QC
Injector carryover	No carryover was observed
Long term storage stability	Confirmed up to 138 days Bias (accuracy) 3% for Low QC, 2% for Med QC, and 8% for High QC

* Low bias = high accuracy

Table S7. Validation Summary Table for Morphine-6-β-D-Glucuronide

Method Description	
Short description of method	Solid-phase extraction with reverse-phase HPLC with MS/MS detection
Matrix	Mouse serum
Analyte	Morphine-6-β-D-Glucuronide
Internal standard (IS)	Morphine-6-β-D-Glucuronide-d3 (deuterated Morphine-6-β-D-Glucuronide)
Calibration concentrations	50 ng/mL to 5000 ng/mL
QC concentrations	50 ng/mL (LLOQ), 150 ng/mL (Low QC), 400 ng/mL (Med QC), and 4000 ng/mL (High QC)
Selectivity	No peaks
Lower limit of quantification	50 ng/mL Between-run absolute bias (accuracy): 10% Between-run precision: 8% Within-run absolute bias (accuracy): 8-13% Within-run precision: 3-11%
Between-run absolute bias (accuracy)*	1-10%
Between-run precision	8-14%
Within-run absolute bias (accuracy)*	Run 1: 4-11% Run 2: 8-14% Run 3: 1-9%
Within-run precision	Run 1: 4-11% Run 2: 5-6% Run 3: 6-11%
Matrix effect	Low QC: 26% (16% CV) High QC: 15% (3% CV)
Recovery of analyte	52-62%
Recovery of IS	59%
Auto-sampler storage stability	Confirmed for up to 72 hr at 40°C nominal 24 hr: Bias (accuracy) 8% at LLOQ, 1% at Low QC, 2% at Med QC, and 5% at High QC 48 hr: Bias (accuracy) 11% at LLOQ, 2% at Low QC, 10% at Med QC, and 1% at High QC 72 hr: Bias (accuracy) 11% at LLOQ, 21% at Low QC, 10% at Med QC, and 8% at High QC
Freeze-thaw stability	Confirmed up to 3 cycles Bias (accuracy) 2% for Low QC and 2% for High QC
Bench top stability	Confirmed up to 24 hr at room temperature Bias (accuracy) 8% for Low QC and 2% for High QC
Injector carryover	No carryover was observed**
Long term storage stability	Confirmed up to 138 days Bias (accuracy) 8% for Low QC, 11% for Med QC, and 13% for High QC

* Low bias = high accuracy

** Two exceptions: 3660 ng/mL measured in double blank during Run 1; 25.1 ng/mL carryover between Calibrator 7 and blank in 10/19/23 run (spleen/small intestine/large intestine)

Table S8. Absolute Between-Run Accuracy (Bias)

Accuracy (%Bias) (n = 14-15)				
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	11	4.1	7.0	10
Norfentanyl	3.7	1.3	0.46	3.1
4-ANPP	7.5	5.7	10	14
Morphine	4.2	1.0	6.6	10
Morphine-3-β-D-Glucuronide	12	4.4	0.29	3.8
Morphine-6-β-D-Glucuronide	10	2.9	1.4	4.4

LLOQ (lower limit of quantification): 1 ng/mL (fentanyl & 4-ANPP), 5 ng/mL (norfentanyl), 10 ng/mL (morphine), 50 ng/mL (morphine-3-β-D- and -6-β-glucuronide)
Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

% bias = relative bias = ((sample value—reference value)/reference value)(100)

Absolute Within-Run Accuracy (Bias)

Table S9.1. Run 1

Accuracy (%Bias) n = 5				
Analyte	LLOQ*	Low QC	Med QC	High QC
Fentanyl	15	8.8	2.7	14
Norfentanyl	16	16	12	4.1
4-ANPP	7.6	3.5	6.2	8.0
Morphine	2.6	11	0.80	7.0
Morphine-3-β-D-glucuronide	30	6.4	9.0	1.6
Morphine-6-β-D-glucuronide	13	11	11	4.0

* Samples run with separate batch (10/3/23)

Table S9.2. Run 2

Accuracy (%Bias) n = 4-5				
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	16	15	7.6	11
Norfentanyl	3.5	11	12	13
4-ANPP	16	20	15	18
Morphine	10	10	10	15
Morphine-3-β-D-glucuronide	11	19	18	8.8
Morphine-6-β-D-glucuronide	7.8	14	14	12

Table S9.3. Run 3

Accuracy (%Bias) n = 5				
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	2.3	6.5	11	5.0
Norfentanyl	8.8	8.7	4.3	2.8
4-ANPP	0.56	0.53	8.8	18
Morphine	5.2	4.6	10	10
Morphine-3-β-D-glucuronide	5.2	0.13	6.3	2.1
Morphine-6-β-D-glucuronide	8.8	6.0	5.0	1.5

LLOQ (lower limit of quantification): 1 ng/mL (fentanyl & 4-ANPP), 5 ng/mL (norfentanyl), 10 ng/mL (morphine), 50 ng/mL (morphine-3-β-D- and -6-β-glucuronide)

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Table S10. Absolute Between-Run Precision

	Mean ± SD ng/mL (%CV) (n = 14-15)			
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	1.1 ± 0.17 (15)	3.1 ± 0.50 (16)	8.0 ± 0.52 (6.5)	82 ± 5.4 (6.6)
Norfentanyl	5.2 ± 0.59 (11)	15 ± 2.3 (15)	40 ± 4.5 (11)	413 ± 36 (8.7)
4-ANPP	1.1 ± 0.10 (10)	3.2 ± 0.41 (13)	8.2 ± 0.53 (6.4)	86 ± 5.5 (6.4)
Morphine	10 ± 1.3 (13)	30 ± 4.0 (13)	80 ± 6.3 (7.8)	826 ± 42 (5.1)
Morphine-3-β-D-glucuronide	56 ± 8.4 (15)	157 ± 18 (11)	399 ± 55 (14)	4152 ± 284 (6.9)
Morphine-6-β-D-glucuronide	55 ± 4.1 (7.5)	154 ± 21 (14)	394 ± 47 (12)	4177 ± 322 (7.7)

LLOQ (lower limit of quantification): 1 ng/mL (fentanyl & 4-ANPP), 5 ng/mL (norfentanyl), 10 ng/mL (morphine), 50 ng/mL (morphine-3-β-D- and -6-β-D-glucuronide)

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Absolute Within-Run Precision

Table S11.1. Run 1

	Mean ± SD ng/mL (%CV) (n = 5)			
Analyte	LLOQ*	Low QC	Med QC	High QC
Fentanyl	1.1 ± 0.051 (4.5)	2.7 ± 0.43 (16)	7.7 ± 0.53 (6.9)	85 ± 3.6 (4.2)
Norfentanyl	5.8 ± 0.12 (2.1)	13 ± 2.3 (18)	35 ± 1.5 (4.2)	384 ± 24 (6.2)
4-ANPP	1.1 ± 0.062 (5.8)	2.9 ± 0.40 (14)	8.0 ± 0.31 (3.9)	81 ± 4.4 (5.4)
Morphine	9.7 ± 1.9 (19)	27 ± 4.8 (18)	76 ± 3.9 (5.1)	803 ± 42 (5.3)
Morphine-3-β-D-glucuronide	645 ± 3.2 (5.0)	140 ± 7.5 (5.3)	364 ± 36 (10)	4062 ± 182 (4.5)
Morphine-6-β-D-glucuronide	57 ± 1.5 (2.6)	133 ± 15 (11)	358 ± 14 (4.0)	4158 ± 231 (5.6)

*Samples run with separate batch (10/3/23)

Table S11.2. Run 2

	Mean ± SD ng/mL (%CV) (n = 4-5)			
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	1.2 ± 0.26 (22)	3.4 ± 0.34 (10)	8.1 ± 0.32 (4.0)	83 ± 2.5 (3.0)
Norfentanyl	5.2 ± 0.39 (7.5)	17 ± 0.81 (4.9)	45 ± 2.5 (5.6)	450 ± 21 (4.7)
4-ANPP	1.2 ± 0.084 (7.3)	3.6 ± 0.19 (5.3)	8.7 ± 0.24 (2.8)	88 ± 1.2 (1.4)
Morphine	11 ± 0.54 (4.9)	33 ± 1.3 (3.9)	82 ± 6.7 (8.1)	861 ± 20 (2.3)
Morphine-3-β-D-glucuronide	55 ± 4.2 (7.6)	179 ± 2.2 (1.2)	473 ± 9.6 (2.0)	4353 ± 343 (7.9)
Morphine-6-β-D-glucuronide	54 ± 3.4 (6.4)	171 ± 9.5 (5.5)	458 ± 25 (5.5)	4495 ± 229 (5.1)

Table S11.3. Run 3

	Mean ± SD ng/mL (%CV) (n = 5)			
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	1.0 ± 0.043 (4.2)	3.2 ± 0.45 (14)	8.3 ± 0.46 (5.5)	79 ± 6.6 (8.3)
Norfentanyl	4.6 ± 0.28 (6.1)	16 ± 0.55 (3.4)	42 ± 1.8 (4.3)	411 ± 27 (6.5)
4-ANPP	0.99 ± 0.093 (9.3)	3.0 ± 0.16 (5.4)	8.2 ± 0.64 (7.9)	89 ± 5.3 (6.0)
Morphine	11 ± 0.76 (7.2)	31 ± 2.0 (6.4)	82 ± 5.5 (6.7)	822 ± 37 (4.6)
Morphine-3-β-D-glucuronide	47 ± 5.6 (12)	150 ± 6.9 (4.6)	375 ± 32 (8.5)	4082 ± 232 (5.7)
Morphine-6-β-D-glucuronide	54 ± 5.8 (11)	159 ± 15 (9.4)	380 ± 28 (7.3)	3942 ± 241 (6.1)

LLOQ (lower limit of quantification): 1 ng/mL (fentanyl & 4-ANPP), 5 ng/mL (norfentanyl), 10 ng/mL (morphine), 50 ng/mL (morphine-3-β-D- and -6-β-D-glucuronide)

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Table S12. Recovery of Analytes

	%Recovery (n = 5)	
Analyte	Low QC	High QC
Fentanyl	66	93
Norfentanyl	101	139
4-ANPP	69	88
Morphine	77	90
Morphine-3-β-D-glucuronide	66	81
Morphine-6-β-D-glucuronide	52	62

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide

Table S13. Recovery of Internal Standards

Analyte	%Recovery (n = 10)
Fentanyl-d5	93
Norfentanyl-d5	118
4-ANPP-d5	85
Morphine-d3	93
Morphine-3-β-D-glucuronide-d3	84
Morphine-6-β-D-glucuronide-d3	59

Table S14. Matrix Effect: Standard

	Matrix Effect (n = 5)	
Analyte	Low QC	High QC
Fentanyl	45	15
Norfentanyl	15	5.7
4-ANPP	68	14
Morphine	19	11
Morphine-3-β-D-glucuronide	26	12
Morphine-6-β-D-glucuronide	26	15

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Table S15. Matrix Effect: Internal Standard

Analyte	Matrix Effect (n = 10)
Fentanyl-d5	22
Norfentanyl-d5	8.7
4-ANPP-d5	25
Morphine-d3	13
Morphine-3-β-D-glucuronide-d3	14
Morphine-6-β-D-glucuronide-d3	17

Table S16. Analyte Stability under Different Storage Conditions

		Mean ± SD ng/mL (%CV) (n = 5)	
Analyte	Stability Test	Low QC	High QC
Fentanyl	Benchtop	2.9 ± 0.38 (13)	82 ± 6.9 (8.5)
	Freeze/Thaw	3.1 ± 0.38 (12)	84 ± 5.6 (6.7)
Norfentanyl	Benchtop	12 ± 1.2 (10)	402 ± 32 (8.0)
	Freeze/Thaw	13 ± 1.2 (8.8)	421 ± 30 (7.2)
4-ANPP	Benchtop	2.8 ± 0.14 (4.9)	84 ± 4.5 (5.4)
	Freeze/Thaw	2.9 ± 0.61 (21)	81 ± 5.9 (7.3)
Morphine	Benchtop	33 ± 2.5 (7.8)	829 ± 57 (6.9)
	Freeze/Thaw	34 ± 1.6 (4.9)	836 ± 38 (4.6)
Morphine-3-β-D-glucuronide	Benchtop	159 ± 15 (9.5)	4620 ± 414 (9.0)
	Freeze/Thaw	175 ± 6.9 (4.0)	4692 ± 239 (5.1)
Morphine-6-β-D-glucuronide	Benchtop	137 ± 8.2 (6.0)	4086 ± 361 (8.8)
	Freeze/Thaw	152 ± 6.9 (4.6)	4082 ± 227 (5.6)

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Analyte Stability Over Time
Table S17.1. 24 hr post-preparation

	Mean ± SD ng/mL (%CV) (n = 5)			
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	0.92 ± 0.033 (3.6)	2.9 ± 0.30 (10)	8.1 ± 0.71 (8.8)	79 ± 8.9 (11)
Norfentanyl	4.4 ± 0.14 (3.1)	16 ± 0.44 (2.8)	41 ± 2.5 (6.1)	435 ± 20 (4.5)
4-ANPP	0.99 ± 0.10 (10)	2.9 ± 0.16 (5.4)	8.0 ± 0.86 (11)	84 ± 7.8 (9.3)
Morphine	10 ± 1.2 (12)	32 ± 1.1 (3.5)	85 ± 2.8 (3.3)	839 ± 38 (4.5)
Morphine-3-β-D-glucuronide	48 ± 4.5 (9.3)	150 ± 17 (11)	386 ± 14 (3.8)	3866 ± 328 (8.5)
Morphine-6-β-D-glucuronide	54 ± 5.8 (11)	152 ± 20 (13)	393 ± 17 (4.4)	3814 ± 251 (6.6)

Table S17.2. 48 hr post-preparation

	Mean ± SD ng/mL (%CV) (n = 4-5)			
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	1.1 ± 0.055 (5.1)	2.8 ± 0.17 (5.9)	7.9 ± 0.53 (6.7)	83 ± 4.2 (5.1)
Norfentanyl	4.6 ± 0.30 (6.6)	16 ± 0.66 (4.2)	41 ± 2.0 (4.9)	431 ± 23 (5.3)
4-ANPP	0.90 ± 0.11 (12)	3.2 ± 0.38 (12)	7.9 ± 0.38 (5)	90 ± 4.7 (5.2)
Morphine	11 ± 1.1 (11)	32 ± 2.7 (8.2)	84 ± 2.7 (3.2)	812 ± 66 (8.2)
Morphine-3-β-D-glucuronide	51 ± 4.8 (9.4)	159 ± 20 (13)	436 ± 23 (5.3)	3970 ± 460 (12)
Morphine-6-β-D-glucuronide	56 ± 5.7 (10)	153 ± 15 (10)	441 ± 26 (5.9)	441 ± 26 (5.9)

Table S17.3. 72 hr post-preparation

	Mean ± SD ng/mL (%CV) (n = 5)			
Analyte	LLOQ	Low QC	Med QC	High QC
Fentanyl	1.0 ± 0.079 (7.9)	3.1 ± 0.18 (5.7)	8.2 ± 0.60 (7.3)	76 ± 5.7 (7.4)
Norfentanyl	5.3 ± 0.55 (10)	17 ± 0.45 (2.6)	42 ± 2.3 (5.5)	421 ± 15 (3.5)
4-ANPP	1.0 ± 0.11 (11)	3.3 ± 0.41 (13)	8.9 ± 0.75 (8.4)	84 ± 4.8 (5.7)
Morphine	10 ± 1.4 (14)	33 ± 2.4 (7.1)	84 ± 5.9 (7.1)	825 ± 62 (7.6)
Morphine-3-β-D-glucuronide	55 ± 5.4 (10)	177 ± 17 (9.4)	447 ± 21 (4.8)	4262 ± 268 (6.3)
Morphine-6-β-D-glucuronide	55 ± 3.6 (6.6)	182 ± 4.1 (2.3)	440 ± 13 (2.9)	4316 ± 369 (8.6)

LLOQ (lower limit of quantification): 1 ng/mL (fentanyl & 4-ANPP), 5 ng/mL (norfentanyl), 10 ng/mL (morphine), 50 ng/mL (morphine-3-β-D- and -6-β-D-glucuronide)
Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Absolute Accuracy (Bias) for Analyte Stability Over Time
Table S18.1. 24 hr post-preparation

Analyte	LLOQ	Low QC	Med QC	High QC
	Accuracy (%Bias) (n = 5)			
Fentanyl	7.6	3.5	7.5	5.2
Norfentanyl	11	5.9	1.9	8.7
4-ANPP	1.0	4.0	7.1	11
Morphine	1.1	7.2	14	12
Morphine-3-β-D-glucuronide	4.0	0.27	3.5	3.4
Morphine-6-β-D-glucuronide	7.7	1.5	1.8	4.7

Table S18.2. 48 hr post-preparation

Analyte	LLOQ	Low QC	Med QC	High QC
	Accuracy (%Bias) (n = 4-5)			
Fentanyl	6.6	6.7	5.0	10
Norfentanyl	7.2	5.9	2.7	7.7
4-ANPP	10	5.2	4.9	19
Morphine	7.5	7.6	12	8.3
Morphine-3-β-D-glucuronide	1.4	5.7	9.1	0.75
Morphine-6-β-D-glucuronide	11	1.7	10	0.70

Table S18.3. 72 hr post-preparation

Analyte	LLOQ	Low QC	Med QC	High QC
	Accuracy (%Bias) (n = 5)			
Fentanyl	0.58	3.1	10	1.9
Norfentanyl	6.0	15	5.2	5.2
4-ANPP	4.0	8.4	18	13
Morphine	0.58	10	12	10
Morphine-3-β-D-glucuronide	10	18	12	6.6
Morphine-6-β-D-glucuronide	11	21	10	7.9

LLOQ (lower limit of quantification): 1 ng/mL (fentanyl & 4-ANPP), 5 ng/mL (norfentanyl), 10 ng/mL (morphine), 50 ng/mL (morphine-3-β-D- and -6-β-D-glucuronide)
Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)
High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Table S19.1. Fentanyl Quality Controls for 13 Experimental Matrices

Sample type (n = 3)	Mean ± SD ng/mL or ng/g (%CV)		
	Low QC	Med QC	High QC
Whole blood	3.2 ± 0.30 (9.4)	7.5 ± 0.72 (10)	80 ± 5.7 (7.2)
Brain	3.4 ± 0.039 (1.1)	7.9 ± 0.35 (4.4)	75 ± 5.4 (7.2)
Liver	3.2 ± 0.062 (1.9)	7.7 ± 0.35 (4.5)	69 ± 1.3 (1.8)
Heart	3.2 ± 0.066 (2.1)	8.4 ± 0.37 (4.4)	78 ± 7.1 (9.1)
Lung	3.3 ± 0.091 (2.8)	8.2 ± 0.42 (5.2)	76 ± 8.9 (12)
Kidney	3.4 ± 0.045 (1.3)	7.8 ± 0.80 (10)	76 ± 7.2 (10)
Spleen	2.9 ± 0.32 (11)	7.7 ± 0.35 (4.5)	80 ± 1.2 (1.6)
Small Intestine	3.02 ± 0.16 (5.1)	8.1 ± 0.42 (5.2)	80 ± 3.5 (4.4)
Large Intestine	3.0 ± 0.29 (10)	8.5 ± 0.87 (10)	72 ± 2.2 (3.1)
Stomach	3.4 ± 0.045 (1.3)	7.9 ± 0.24 (3.0)	77 ± 6.5 (8.4)
Muscle	3.3 ± 0.017 (0.51)	8.1 ± 0.25 (3.0)	82 ± 1.8 (2.3)
Fat	3.3 ± 0.054 (1.6)	8.3 ± 0.052 (0.63)	85 ± 0.52 (0.62)
Skin	2.7 ± 0.093 (3.5)	7.4 ± 0.49 (6.6)	79 ± 1.1 (1.3)

Table S19.2. Norfentanyl Quality Controls for 13 Experimental Matrices

Sample type (n = 3)	Mean ± SD ng/mL or ng/g (%CV)		
	Low QC	Med QC	High QC
Whole blood	13 ± 0.16 (1.2)	42 ± 2.5 (6.1)	431 ± 29 (6.7)
Brain	16 ± 0.71 (4.6)	38 ± 2.5 (6.5)	426 ± 11 (2.7)
Liver	14 ± 1.2 (8.7)	34 ± 0.78 (2.3)	344 ± 13 (3.8)
Heart	16 ± 2.5 (16)	45 ± 2.6 (5.8)	399 ± 23 (5.8)
Lung	14 ± 0.53 (3.8)	42 ± 4.5 (11)	358 ± 14 (3.9)
Kidney	15 ± 2.3 (16)	42 ± 4.8 (11)	409 ± 5.6 (1.4)
Spleen	16 ± 2.2 (14)	40 ± 1.1 (2.6)	405 ± 14 (3.4)
Small Intestine	18 ± 0.68 (3.9)	42 ± 0.24 (0.59)	414 ± 18 (4.4)
Large Intestine	17 ± 1.2 (7.3)	41 ± 0.14 (0.34)	366 ± 8.3 (2.3)
Stomach	18 ± 0.39 (2.2)	39 ± 1.2 (3.0)	392 ± 2.4 (0.60)
Muscle	15 ± 0.42 (2.8)	37 ± 0.19 (0.50)	391 ± 5.9 (1.5)
Fat	18 ± 0.42 (2.3)	41 ± 0.45 (1.1)	435 ± 5.9 (1.4)
Skin	13 ± 0.17 (1.4)	36 ± 1.7 (4.8)	403 ± 2.6 (0.65)

Table S19.3. 4-ANPP Quality Controls for 13 Experimental Matrices

Sample type (n = 3)	Mean ± SD ng/mL or ng/g (%CV)		
	Low QC	Med QC	High QC
Whole blood	3.1 ± 0.31 (10)	8.2 ± 0.22 (2.9)	77 ± 2.1 (2.8)
Brain	3.1 ± 0.21 (6.6)	8.7 ± 0.071 (0.82)	78 ± 11 (14)
Liver	3.0 ± 0.16 (5.4)	8.4 ± 0.38 (4.5)	74 ± 1.4 (1.9)
Heart	3.1 ± 0.31 (10)	8.6 ± 0.71 (8.2)	68 ± 2.1 (3.0)
Lung	3.3 ± 0.59 (18)	8.6 ± 0.33 (3.8)	71 ± 2.0 (2.8)
Kidney	3.0 ± 0.22 (7.5)	8.3 ± 0.37 (4.5)	83 ± 4.5 (5.5)
Spleen	2.7 ± 0.24 (9.1)	7.5 ± 0.16 (2.2)	71 ± 3.4 (4.8)
Small Intestine	3.0 ± 0.034 (1.1)	7.9 ± 0.016 (0.21)	82 ± 0.33 (0.40)
Large Intestine	3.7 ± 0.66 (18)	7.6 ± 0.19 (2.5)	69 ± 2.1 (3.0)
Stomach	3.1 ± 0.12 (3.7)	7.5 ± 0.26 (3.5)	75 ± 5.2 (7.0)
Muscle	3.0 ± 0.071 (2.3)	7.8 ± 0.25 (3.2)	74 ± 2.6 (3.5)
Fat	3.4 ± 0.18 (5.3)	7.5 ± 0.34 (4.6)	81 ± 1.2 (1.5)
Skin	2.5 ± 0.11 (4.4)	6.8 ± 0.12 (1.7)	73 ± 1.5 (2.0)

Table S19.4. Morphine Quality Controls for 13 Experimental Matrices

Sample type (n = 1-3)	Low QC	Med QC	High QC
Whole blood	31 ± 3.1 (10)	76 ± 6.8 (8.8)	774 ± 88 (11)
Brain	34 ± 6.4 (19)	75 ± 4.5 (6.0)	794 ± 41 (5.1)
Liver	34 ± 2.2 (6.5)	73 ± 3.2 (4.3)	719 ± 33 (4.6)
Heart	27 ± 2.2 (8.0)	77 ± 0.94 (1.2)	735 ± 0 (0)
Lung	31±0 (0)		860 ±6.1 (0.71)
Kidney	33 ± 0 (0)	84 ± 0 (0)	800 ± 49 (6.1)
Spleen	31 ± 1.5 (4.9)	84 ± 4.7 (5.5)	775 ± 49 (6.4)
Small Intestine	30 ± 2.2 (7.3)	87 ± 5.7 (6.6)	758 ± 27 (3.5)
Large Intestine	31 ± 0.050 (0.16)	87 ± 8.4 (10)	699 ± 4.8 (0.68)
Stomach	35 ± 0.67 (1.9)	85 ± 0.54 (0.64)	811 ± 13 (1.6)
Muscle			658 ± 48 (7.3)
Fat	35 ± 0.24 (0.70)	86 ± 2.7 (3.2)	875 ± 12 (1.4)
Skin	31 ± 1.4 (4.4)	87 ± 0 (0)	821 ± 10 (1.2)

Mean ± SD ng/mL or ng/g (%CV)

Blank spaces denote QCs rejected due to deviation from acceptable levels of accuracy

Table S19.5. Morphine-3-β-D-Glucuronide Quality Controls for 13 Experimental Matrices

Sample type (n = 3)	Low QC	Med QC	High QC
Whole blood	155 ± 8.7 (5.6)	451 ± 27 (5.9)	4513 ± 496 (11)
Brain	165 ± 9.9 (6.0)	443 ± 20 (4.6)	4367 ± 249 (5.7)
Liver	176 ± 8.2 (4.7)	391 ± 52 (13)	3680 ± 151 (4.1)
Heart	163 ± 20 (12)	410 ± 47 (12)	3547 ± 441 (12)
Lung	167 ± 7.4 (4.4)	440 ± 35 (8.0)	4440 ± 418 (9.4)
Kidney	173 ± 17 (10)	402 ± 57 (14)	4237 ± 204 (4.8)
Spleen	151 ± 23 (15)	386 ± 13 (3.4)	3900 ± 270 (6.9)
Small Intestine	151 ± 9.3 (6.1)	399 ± 32 (7.9)	4270 ± 393 (9.2)
Large Intestine	184 ± 35 (19)	418 ± 4.6 (1.1)	3943 ± 259 (6.6)
Stomach	161 ± 12 (7.2)	412 ± 33 (8.0)	4310 ± 139 (3.2)
Muscle	172 ± 6.2 (3.6)	439 ± 29 (6.6)	4407 ± 62 (1.4)
Fat	164 ± 6.2 (3.8)	422 ± 28 (6.7)	4627 ± 175 (3.8)
Skin	138 ± 4.8 (3.5)	396 ± 11 (2.7)	4723 ± 117 (2.5)

Mean ± SD ng/mL or ng/g (%CV)

Table S19.6. Morphine-6-β-D-Glucuronide Quality Controls for 13 Experimental Matrices

Mean ± SD ng/mL or ng/g (%CV)			
Sample type (n = 1-3)	Low QC	Med QC	High QC
Whole blood	174 ± 6.5 (3.7)	406 ± 49 (12)	4110 ± 200 (4.9)
Brain	169 ± 5.0 (2.9)	390 ± 12 (3.1)	4313 ± 46 (1.1)
Liver	160 ± 9.5 (5.9)	372 ± 25 (6.6)	3994 ± 307 (7.7)
Heart	150 ± 5.3 (3.5)	372 ± 5.9 (1.6)	3767 ± 254 (6.7)
Lung	132 ± 8.3 (6.3)	369 ± 24 (6.6)	4067 ± 171 (4.2)
Kidney	165 ± 16 (10)	429 ± 36 (8.4)	4090 ± 410 (10)
Spleen	133 ± 0 (0)	431 ± 14 (3.4)	4170 ± 128 (3.1)
Small Intestine	142 ± 10 (7.2)	419 ± 26 (6.2)	4070 ± 99 (2.4)
Large Intestine	144 ± 6.9 (4.8)	410 ± 10 (2.5)	3903 ± 296 (7.6)
Stomach	177 ± 1.7 (1.0)	442 ± 16 (3.6)	4457 ± 270 (6.0)
Muscle	163 ± 4.8 (2.9)	396 ± 11 (2.8)	4590 ± 134 (2.9)
Fat	176 ± 2.1 (1.2)	457 ± 3.8 (0.82)	4563 ± 152 (3.3)
Skin	136 ± 2.9 (2.2)	370 ± 19 (5.0)	4000 ± 29 (0.74)

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Table S20.1. Fentanyl: Absolute Accuracy (Bias) in 13 Matrices

	Accuracy (%Bias) n = 3		
Tissue	Low QC	Med QC	High QC
Whole Blood	5.7	0.62	6.8
Brain	14	5.2	0.044
Liver	6.4	2.9	8.0
Heart	5.1	12	4.0
Lung	9.4	10	0.76
Kidney	14	4.5	1.2
Spleen	2.8	3.3	6.2
Small Intestine	0.67	7.7	7.0
Large Intestine	1.0	13	4.5
Stomach	14	5.7	3.3
Muscle	10	7.6	9.1
Fat	10	11	13
Skin	11	1.0	5.1

Table S20.2. Norfentanyl: Absolute Accuracy (Bias) in 13 Matrices

	Accuracy (%Bias) n = 3		
Tissue	Low QC	Med QC	High QC
Whole Blood	11	4.0	7.8
Brain	4.0	5.1	6.6
Liver	6.2	15	14
Heart	4.2	11	0.25
Lung	7.8	5.4	10
Kidney	2.2	5.2	2.2
Spleen	7.6	0.92	1.2
Small Intestine	17	4.2	3.5
Large Intestine	13	2.8	8.6
Stomach	18	2.4	2.1
Muscle	0.67	6.4	2.2
Fat	22	1.3	8.7
Skin	16	10	0.67

Table S20.3. 4-ANPP: Absolute Accuracy (Bias) in 13 Matrices

	Accuracy (%Bias) n = 3		
Tissue	Low QC	Med QC	High QC
Whole Blood	2.9	9.1	2.7
Brain	4.4	16	4.5
Liver	1.3	12	1.5
Heart	4.9	15	9.4
Lung	10	15	5.2
Kidney	1.1	11	10
Spleen	11	0.62	4.7
Small Intestine	0.89	4.8	10
Large Intestine	25	2.0	8.1
Stomach	4.9	0	0.44
Muscle	1.3	3.4	1.8
Fat	14	0.089	7.6
Skin	18	9.4	2.8

Table S20.4. Morphine: Absolute Bias (Accuracy) in 13 Matrices

	Accuracy (%Bias) n = 1-3		
Tissue	Low QC	Med QC	High QC
Whole Blood	4.3	2.0	3.2
Brain	12	0.36	5.8
Liver	14	2.6	4.2
Heart	10	2.2	2.0
Lung	1.7		15
Kidney	10	13	6.7
Spleen	4.2	12	3.3
Small Intestine	0.78	16	1.1
Large Intestine	3.5	16	6.8
Stomach	16	14	8.2
Muscle			12
Fat	16	15	17
Skin	13	16	19

Blank spaces denote QCs rejected due to deviation from acceptable levels of accuracy

Table S20.5. Morphine-3-β-D-Glucuronide: Absolute Bias (Accuracy) in 13 Matrices

Tissue	Low QC	Med QC	High QC
	Accuracy (%Bias) n = 3		
Whole Blood	3.1	13	13
Brain	10	11	9.2
Liver	18	2.3	8.0
Heart	8.4	2.5	11
Lung	12	10	11
Kidney	16	0.42	5.9
Spleen	0.44	2.5	2.5
Small Intestine	0.67	0.17	6.8
Large Intestine	22	4.6	1.4
Stomach	7.3	2.9	7.8
Muscle	14	10	10
Fat	10	5.5	16
Skin	8.2	0.92	18

Table S20.6. Morphine-6-β-D-Glucuronide: Absolute Bias (Accuracy) in 13 Matrices

Tissue	Low QC	Med QC	High QC
	Accuracy (%Bias) n = 3		
Whole Blood	16	1.4	2.8
Brain	13	2.6	7.8
Liver	6.4	7.0	0.15
Heart	0.22	7.1	5.8
Lung	12	7.7	1.7
Kidney	10	7.3	2.3
Spleen	11	7.7	4.3
Small Intestine	5.3	4.8	1.8
Large Intestine	4.2	2.4	2.4
Stomach	18	11	11
Muscle	8.9	1.1	15
Fat	18	14	14
Skin	9.3	7.5	0

Low QC (low quality control): 3 ng/mL (fentanyl & 4-ANPP), 15 ng/mL (norfentanyl), 30 ng/mL (morphine), 150 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Med QC (medium quality control): 7.5 ng/mL (fentanyl & 4-ANPP), 40 ng/mL (norfentanyl), 75 ng/mL (morphine), 400 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

High QC (high quality control): 75 ng/mL (fentanyl & 4-ANPP), 400 ng/mL (norfentanyl), 750 ng/mL (morphine), 4,000 ng/mL (morphine 3-β-D- and -6-β-D-glucuronide)

Figure S11a demonstrates that, in whole blood, both opioids exhibited a t_{max} of 15 min (**Figure S11a**). Average fentanyl AUC was 3790 ng/mL*min, while average morphine AUC was 1183413 ng/mL*min (p=0.0019). Fentanyl was detected at significantly higher concentrations than its metabolite norfentanyl in blood at 5 and 15 min (p<0.0001), and significantly exceeded 4-ANPP at 5 (p<0.0001), 15 (p<0.0001), and 60 min (p<0.05) (**Figure S11b**). No significant differences were observed between morphine and its metabolite, morphine-3-β-D-glucuronide, at any of the time points studied (**Figure S11c**).
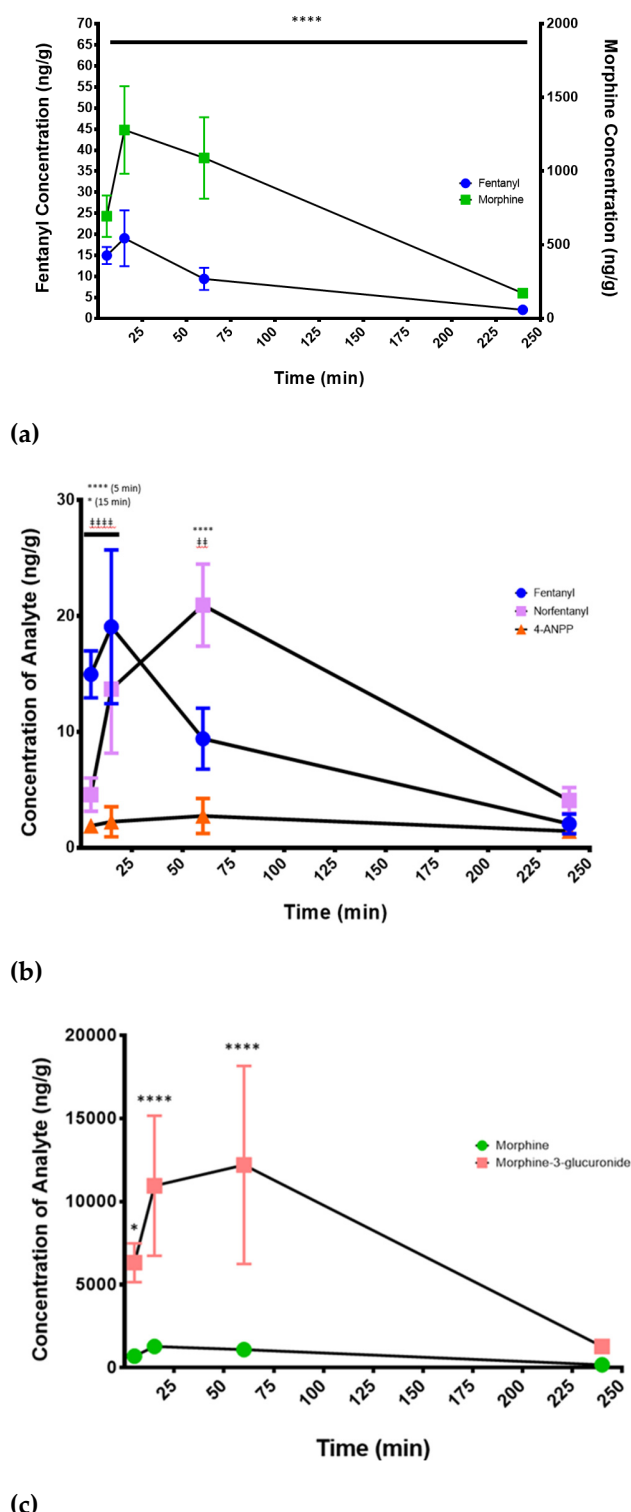
(a)

(b)

(c)

Figure S11. Concentration of Fentanyl, Morphine, and Select Metabolites in Whole Blood from 5-240 Min in Mice.

a) Average whole blood concentration of fentanyl and morphine in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P = 0.0004; F (1, 6) = 49.34), significant effect of time (P<0.0001; F (3, 18) = 20.16), and significant interaction between time and treatment (P<0.0001; F (3, 18) = 19.94). Fentanyl AUC: 3790 ng/mL*min; morphine AUC: 1183413 ng/mL*min. **: p=0.0019

b) Average concentration of fentanyl and its metabolites, norfentanyl and 4-ANPP, in whole blood. Significant difference in analyte concentration (P<0.0001; F (2, 9) = 43.98), significant effect of time (P<0.0001; F (3, 27) = 11.29), and significant interaction of time and analyte concentration (P<0.0001; F (6, 27) = 11.04). ****: p<0.0001 (fentanyl vs norfentanyl); ǂ: p<0.05 (fentanyl vs 4-ANPP); ǂǂǂ: p<0.0001 (fentanyl vs 4-ANPP)

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in whole blood. No significant difference in analyte concentration (P = 0.4525; F (1, 6) = 0.6450), but significant effect of time (P<0.0001; F (3, 18) = 38.98) and significant interaction of time and analyte concentration (P<0.0001; F (3, 18) = 18.14)

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In brain, average fentanyl AUC was 12545 ng/g*min, while average morphine AUC was 294964 ng/g*min (p=0.0001). Over the time points studied, fentanyl t_{max} = 15 min, while morphine exhibited a t_{max} of 60 min (**Figure S12a**). Fentanyl concentrations in brain were greater than norfentanyl at 5 min (p<0.0001), 15 min (p<0.0001), and 60 min (p<0.01) and greater than 4-ANPP at 5 min (p<0.0001), 15 min (p<0.0001), and 60 min (p<0.05) (**Figure S12b**). No significant differences were observed between morphine and morphine-3-β-D-glucuronide in brain at any of the four time points (**Figure S12c**).
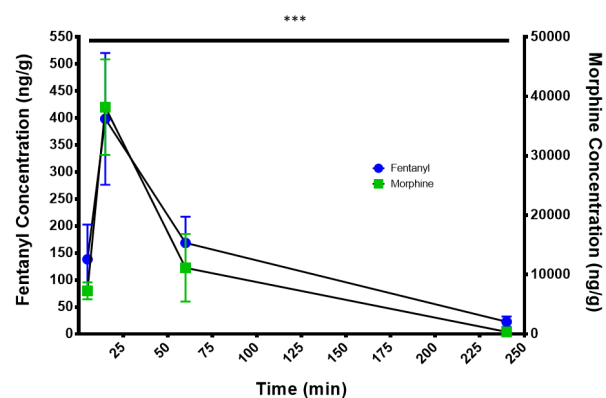
(a)

(b)

(c)

Figure S12. Concentration of Fentanyl, Morphine, and Select Metabolites in Whole Brain from 5-240 Min in Mice.

a) Average fentanyl and morphine concentration in brain in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment ($P<0.0001$; $F_{(1, 6)} = 194.5$), significant effect of time ($P<0.0001$; $F_{(3, 18)} = 17.27$), and significant interaction of time and treatment ($P<0.0001$; $F_{(3, 18)} = 15.37$). Fentanyl AUC: 12545 ng/g*min; morphine AUC: 294964 ng/g*min. ***: $p=0.0001$

b) Average concentration of fentanyl and its metabolites, norfentanyl and 4-ANPP, in brain. Significant difference in analyte concentration ($P = 0.0005$; $F_{(2, 9)} = 80.40$), significant effect of time ($P<0.0001$; $F_{(3, 27)} = 16.01$), and significant interaction of time and analyte concentration ($P<0.0001$; $F_{(6, 27)} = 15.09$). **: $p<0.01$ (fentanyl vs norfentanyl); ****: $p<0.0001$ (fentanyl vs norfentanyl); ǂ: $p<0.05$ (fentanyl vs 4-ANPP); ǂǂǂ: $p<0.0001$ (fentanyl vs 4-ANPP)

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in brain. Significant difference in analyte concentration ($P = 0.0180$; $F_{(1, 6)} = 10.42$ and significant effect of time ($P<0.0001$; $F_{(3, 18)} = 23.00$), but no significant interaction of time and treatment ($P = 0.5934$; $F_{(3, 18)} = 0.6496$).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In liver, average fentanyl AUC was 1882 ng/g*min, while average morphine AUC was 178573 ng/g*min (p<0.0001). Both opioids displayed a t_{max} of 15 min (**Figure S13a**). Fentanyl concentration was greater than norfentanyl at 5 min (p<0.0001) and 15 min (p<0.05), although norfentanyl concentration exceeded fentanyl at 60 min (p<0.0001) (**Figure S13b**). Fentanyl was measured at higher levels than 4-ANPP at 5 min (p<0.0001), 15 min (p<0.0001), and 60 min (p<0.01) (**Figure S13b**). Concentration of morphine-3-β-D-glucuronide exceeded morphine at 5 min (p<0.05), 15 min (p<0.0001), and 60 min (p<0.0001) (**Figure S13c**).
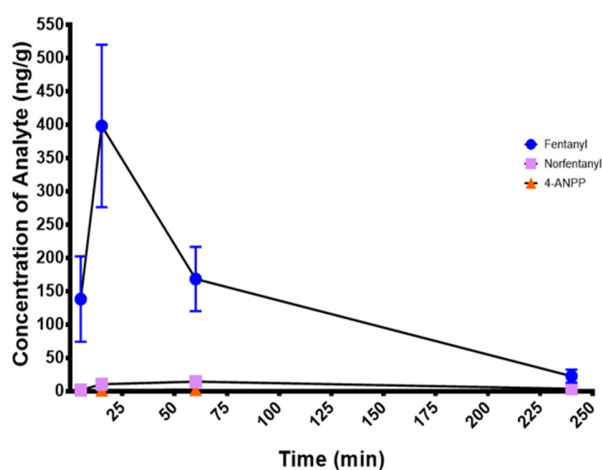
(a)

(b)

(c)

Figure S13. Concentration of Fentanyl, Morphine, and Select Metabolites in Liver from 5-240 Min in Mice.

a) Average fentanyl and morphine concentration in liver in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment ($P<0.0001$; $F_{(1, 6)} = 536.9$), significant effect of time ($P<0.0001$; $F_{(3, 18)} = 17.80$), and significant interaction of time and treatment ($P=0.0231$; $F_{(3, 18)} = 16.97$). fentanyl AUC: 1882 ng/g*min; morphine AUC: 178573 ng/g*min. ****: $p<0.0001$
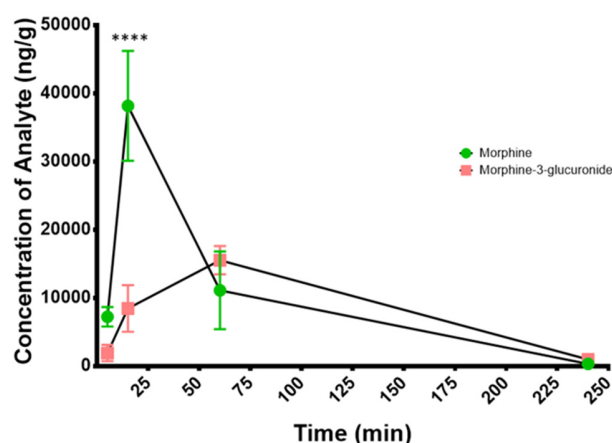
b) Average concentration of fentanyl and its metabolites, norfentanyl and 4-ANPP, in liver. Significant difference in analyte concentration ($P<0.0001$; $F_{(2, 9)} = 75.85$), significant effect of time ($P<0.0001$; $F_{(3, 27)} = 21.43$), and significant interaction of time and analyte concentration ($P<0.0001$; $F_{(6, 27)} = 13.32$). *:$p<0.05$ (fentanyl vs norfentanyl); ****:$p<0.0001$ (fentanyl vs norfentanyl); ⌗: $p<0.01$ (fentanyl vs 4-ANPP); ⌗⌗⌗:$p<0.0001$ (fentanyl vs norfentanyl).

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in liver. Significant difference in analyte concentration ($P<0.001$; $F_{(1, 6)} = 64.17$), significant effect of time ($P = 0.0012$; $F_{(3, 18)} = 8.233$), and significant interaction of time and analyte concentration ($P = 0.0067$; $F_{(3, 18)} = 5.620$). *:$p<0.05$ (morphine vs morphine-3-β-D-glucuronide); ****: $p<0.0001$ (morphine vs morphine-3-β-D-glucuronide).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In lung, average fentanyl AUC was 33028 ng/g*min, while average morphine AUC was 2392610 ng/g*min (p=0.0001). Fentanyl and morphine both exhibited a t_{max} of 15 min (**Figure S14a**). Due to a lack of 4-ANPP data at 5 and 240 min, statistical analysis was unable to be performed on potential differences between fentanyl and its metabolites, although detected quantities of fentanyl appear noticeably higher than norfentanyl and 4-ANPP at all available time points (**Figure S14b**). Concentration of morphine was significantly greater than morphine-3-β-D-glucuronide at 15 min (p<0.0001) (**Figure S14c**).
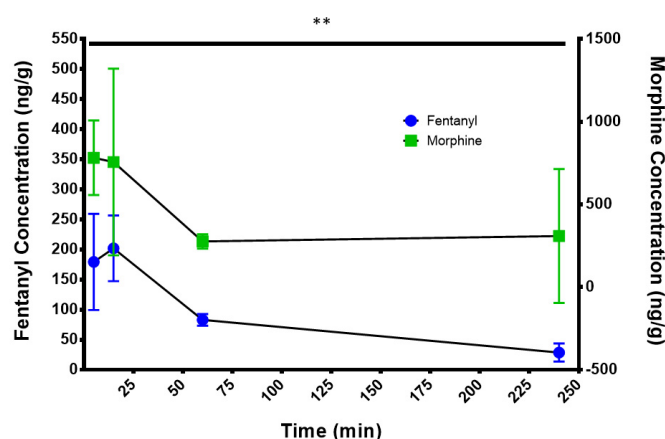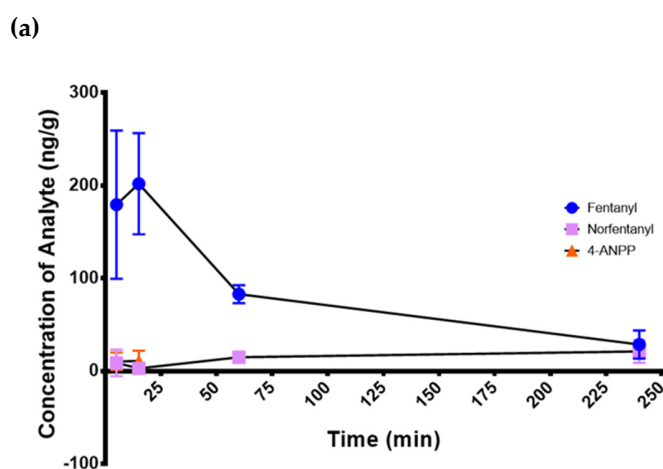
(a)

(b)

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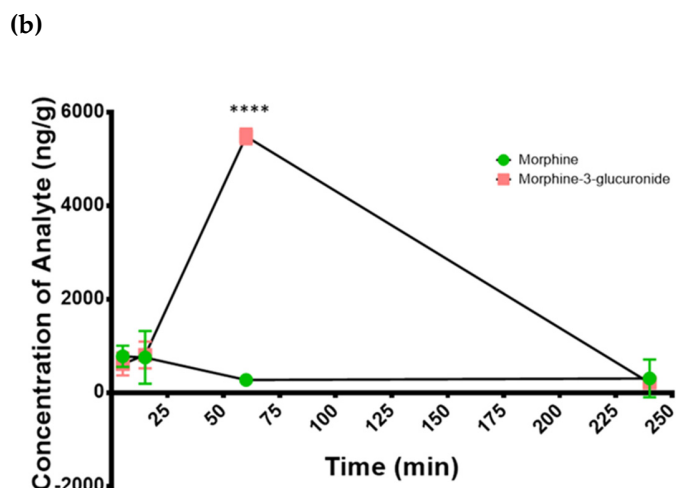**Figure S14. Concentration of Fentanyl, Morphine, and Select Metabolites in Lung from 5-240 Min in Mice.**

a) Average concentration of fentanyl and morphine in lung in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P<0.0001; F (1, 6) = 325.2), significant effect of time (P<0.0001; F (3, 18) = 37.50), and significant interaction of time and treatment (P<0.0001; F (3, 18) = 36.12). Fentanyl AUC: 33028 ng/g*min; morphine AUC: 2392610 ng/g*min. ***: p=0.0001

b) Average concentration of fentanyl and its metabolites, norfentanyl and 4-ANPP, in lung.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in lung. Significant difference in analyte concentration (P = 0.0004; F(1, 6) = 48.05), significant effect of time (P<0.0001; F(3, 18) = 49.64), and significant interaction of time and analyte concentration (P<0.0001; F(3, 18) = 28.79). ****: p<0.0001 (morphine vs morphine-3-β-D-glucuronide).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In heart, average fentanyl AUC was 18806 ng/g*min, while average morphine AUC was 85531 ng/g*min (p=0.0038). Fentanyl exhibited a t_{max} of 15 min, while morphine displayed a t_{max} of 5 min (**Figure S15a**). Due to a lack of 4-ANPP data at 60 min and 240 min, statistical analysis was unable to be performed on potential differences between fentanyl and its metabolites; however, detected quantities of fentanyl appear noticeably higher than norfentanyl at 5 min, 15 min, and 60 min, and higher than 4-ANPP at 5 and 15 min (**Figure S15b**). Morphine levels were significantly lower than morphine-3-β-D-glucuronide at 60 min (p<0.0001) (**Figure S15c**).
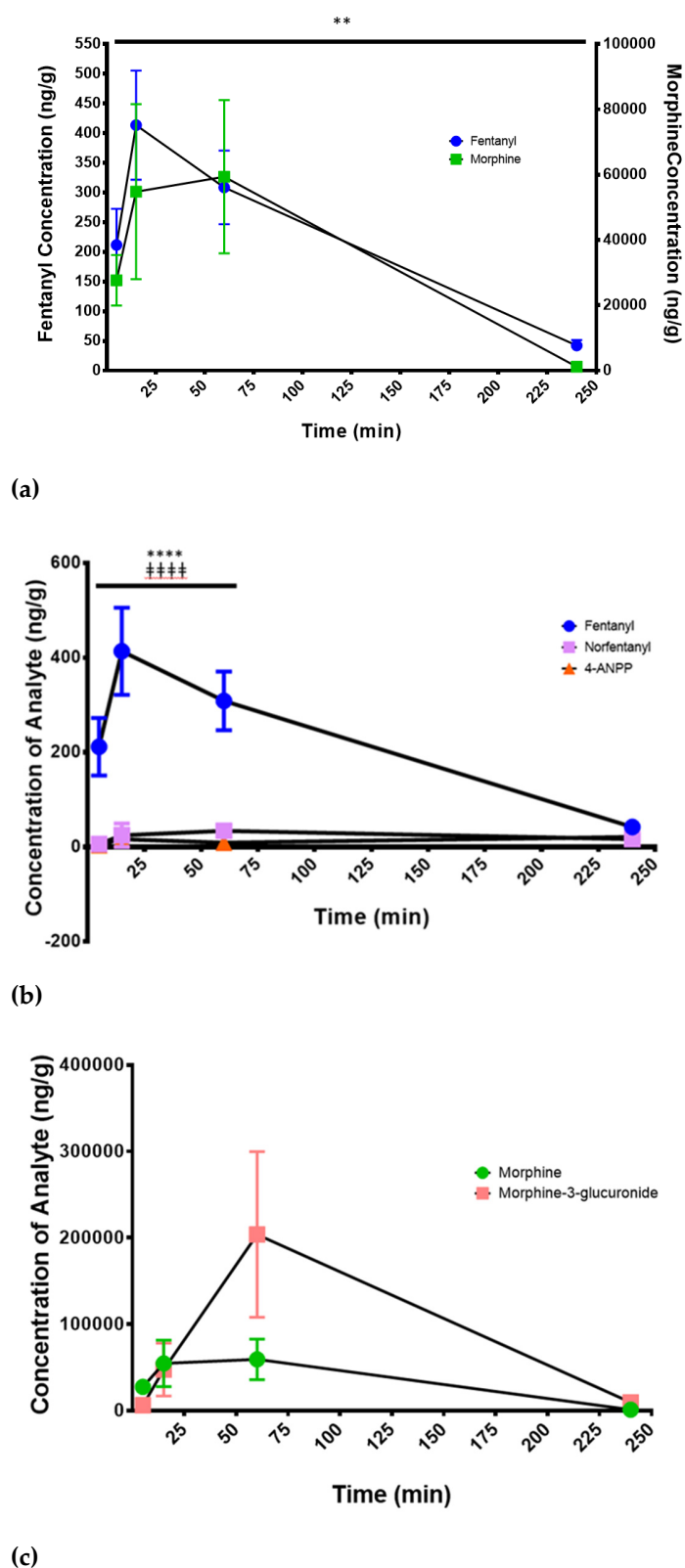
(a)

(b)

(c)

Figure S15. Concentration of Fentanyl, Morphine, and Select Metabolites in Heart from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in heart in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P=0.0018; $F_{(1, 6)} = 28.34$), significant effect of time (P = 0.0435; $F_{(3, 18)} = 3.315$), but no significant interaction of time and treatment (P = 0.3833; $F_{(3, 18)} = 1.078$). Fentanyl AUC: 18806 ng/g*min; morphine AUC: 85531 ng/g*min. **: p=0.0038

b) Average concentration of fentanyl and its metabolites, norfentanyl and 4-ANPP, in heart.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in heart. Significant difference in analyte concentration (P<0.0001; $F_{(1, 6)} = 153.9$), significant effect of time (P<0.0001; $F_{(3, 18)} = 118.7$), and significant interaction of time and treatment (P<0.0001; $F_{(3, 18)} = 150.6$). ****: p<0.0001 (morphine vs morphine-3-β-D-glucuronide)

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In kidney, average fentanyl AUC was 51484 ng/g*min, while average morphine AUC was 8503320 ng/g*min (p=0.0018). Fentanyl exhibited a t_{max} of 15 min, while morphine exhibited a t_{max} of 60 min (**Figure S16a**). Fentanyl was measured at significantly higher levels than its metabolites norfentanyl and 4-ANPP at 5 min (p<0.0001, 15 min (p<0.0001), and 60 min (p<0.0001) (**Figure S16b**). Repeated-measures ANOVA revealed no significant differences between morphine and morphine-3-β-D-glucuronide concentration at any time point (**Figure S16c**).
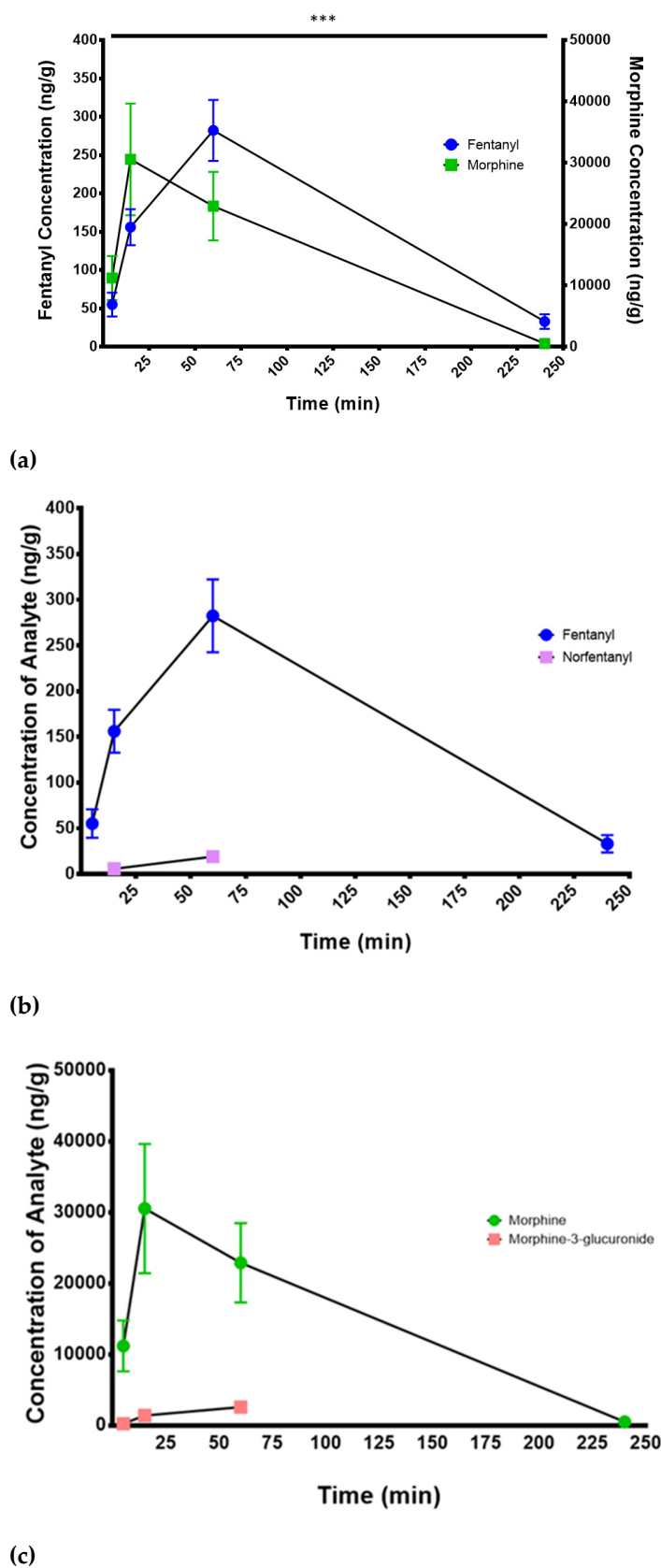
(a)

(b)

(c)

Figure S16. Concentration of Fentanyl, Morphine, and Select Metabolites in Kidney from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in kidney in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P = 0.0011; $F_{(1, 6)}$ = 34.25), significant effect of time (P=0.0002; $F_{(3, 18)}$ = 11.94), and significant interaction of time and treatment (P = 0.0002; $F_{(3, 18)}$ = 11.68). Fentanyl AUC: 51484 ng/g*min; morphine AUC: 8503320 ng/g*min. **: p=0.0018

b) Average concentration of fentanyl and its metabolites, norfentanyl and 4-ANPP, in kidney. Significant difference in analyte concentration (P<0.0001; $F_{(2, 9)}$ = 344.6), significant effect of time (P<0.0001; $F_{(3, 27)}$ = 20.92), and significant interaction of time and analyte concentration (P<0.0001; $F_{(6, 27)}$ = 19.45). ****: p<0.0001 (fentanyl vs norfentanyl); ‡‡‡‡: p<0.0001 (fentanyl vs 4-ANPP)

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glcuronide, in kidney. No significant difference in analyte concentration (P = 0.0627; $F_{(1, 6)}$ = 5.204), but significant effect of time (P<0.0001; $F_{(3, 18)}$ = 18.34) and significant interaction of time and analyte concentration (P<0.01; $F_{(3, 18)}$ = 8.284).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In spleen, average fentanyl AUC was 39463 ng/g*min, while average morphine AUC was 3556074 ng/g*min (p=0.0002). Fentanyl exhibited a t_{max} of 60 min, while morphine's t_{max} occurred at 15 min (**Figure S17a**). Due to a lack of norfentanyl data at 5 and 240 min, statistical analysis could not be performed to compare fentanyl and norfentanyl concentrations, but fentanyl levels appear to be higher relative to norfentanyl at 15 min and 60 min (**Figure S17b**). No 4-ANPP was detected at any time point. Due to a lack of morphine-3-β-D-glucuronide data at 240 min, statistical analysis comparing morphine and morphine-3-β-D-glucuronide levels could not be performed. However, morphine concentration appears noticeably greater than morphine-3-β-D-glucuronide at 5 min, 15 min, and 60 min (**Figure S17c**).
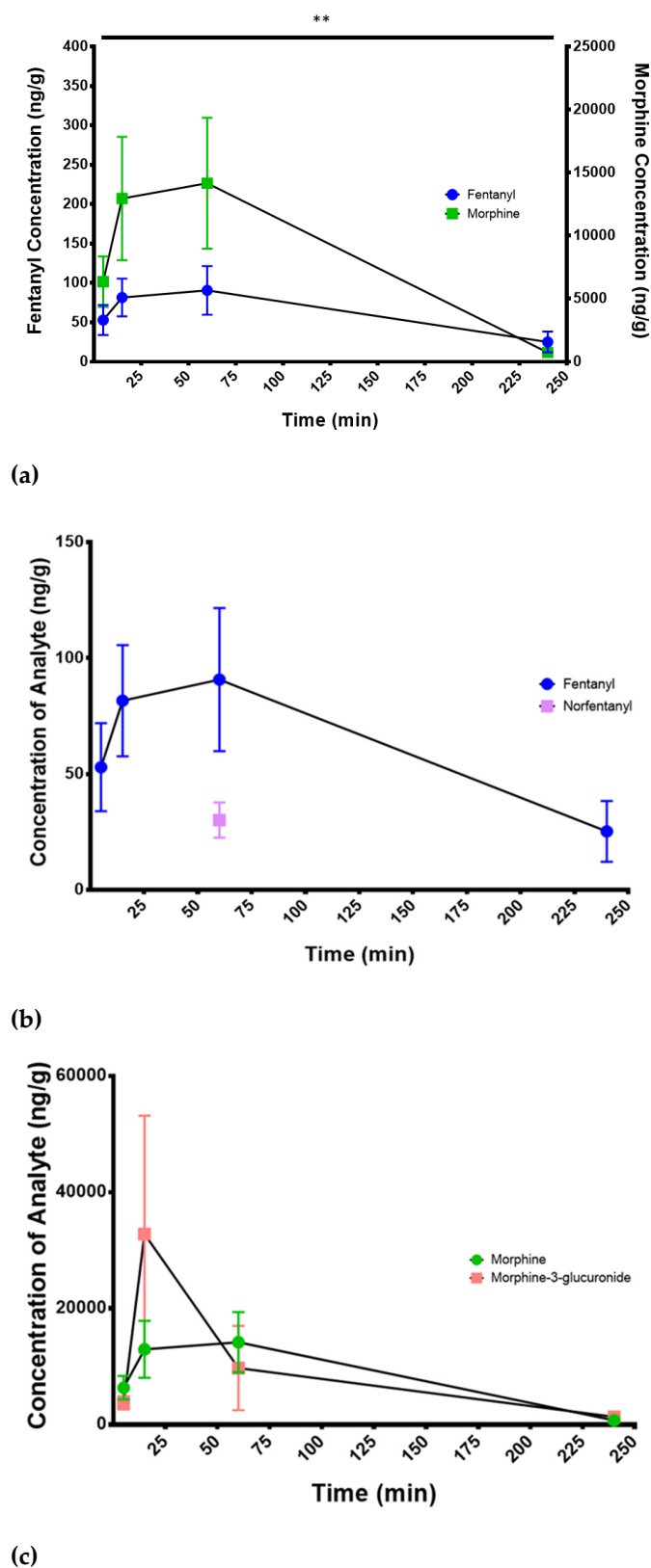
(a)

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(c)

Figure S17. Concentration of Fentanyl, Morphine, and Select Metabolites in Spleen from 5-240 Min in Mice.
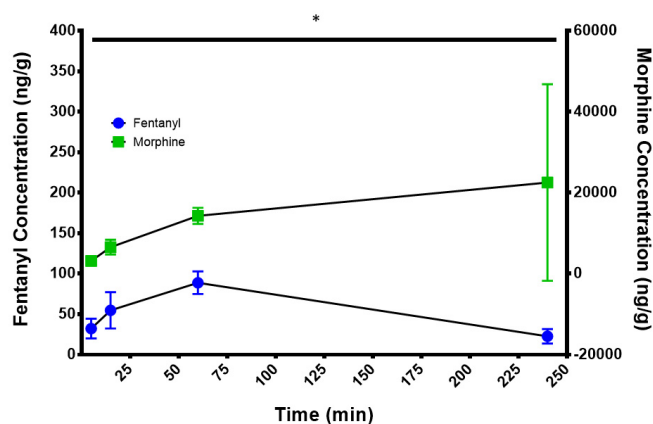
a) Average concentration of fentanyl and morphine in spleen in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P = 0.0002; $F_{(1, 6)}$ = 63.22), significant effect of time (P<0.0001; $F_{(3, 18)}$ = 34.92) and significant interaction of time and treatment (P<0.0001; $F_{(3, 18)}$ = 34.05). Fentanyl AUC: 39463 ng/g*min; morphine AUC: 3556074 ng/g*min. ***: p=0.0002

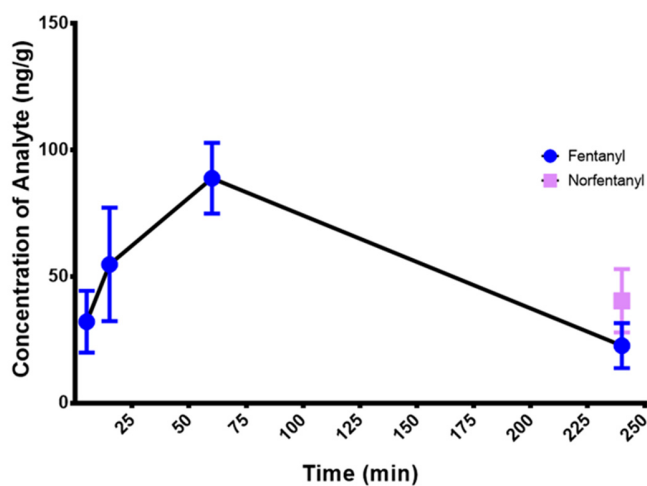b) Average concentration of fentanyl and its metabolite, norfentanyl, in spleen.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in spleen.

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc as needed. n = 4/group

In small intestine, average fentanyl AUC was 15115 ng/g*min, while average morphine AUC was 2064465 ng/g*min (p=0.0010). For both opioids, t_{max} occurred at 60 min (**Figure S18a**). Due to the lack of norfentanyl data at 5 min, 15 min, and 240 min, statistical analysis comparing fentanyl and norfentanyl concentrations could not be performed, but fentanyl appears greater than norfentanyl at 60 min (**Figure S18b**). No 4-ANPP was detected at any time point. Repeated-measures ANOVA revealed no significant differences between morphine and morphine-3-β-D-glucuronide at any time point (**Figure S18c**).
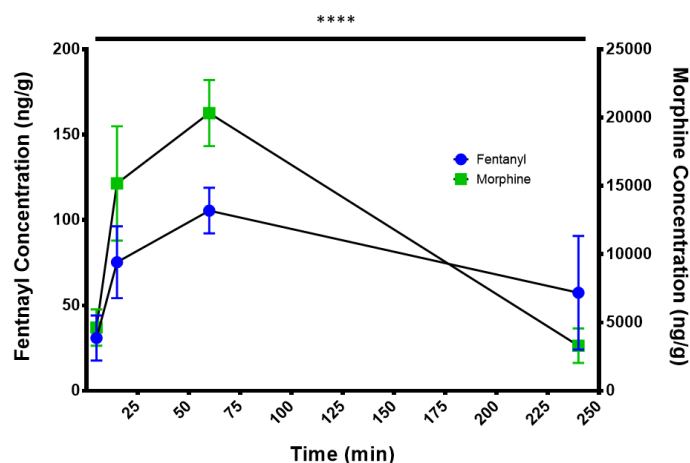
(a)

(b)

(c)

Figure S18. Concentration of Fentanyl, Morphine, and Select Metabolites in Small Intestine from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in small intestine mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P = 0.0005; $F_{(1, 6)}$ = 47.35), significant effect of time (P<0.0001; $F_{(3, 18)}$ = 15.42), and significant interaction of time and treatment (P<0.0001; $F_{(3, 18)}$ = 15.13). Fentanyl AUC: 15115 ng/g*min; morphine AUC: 2064465 ng/g*min. **: p=0.0010

b) Average concentration of fentanyl and its metabolites, norfentanyl, in small intestine.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in small intestine. No significant difference in analyte concentration (P = 0.3344; $F_{(1, 6)}$ = 1.101), but significant effect of time (P = 0.0001; $F_{(3, 18)}$ = 12.13) and significant interaction between time and analyte concentration (P = 0.0212; $F_{(3, 18)}$ = 4.151).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

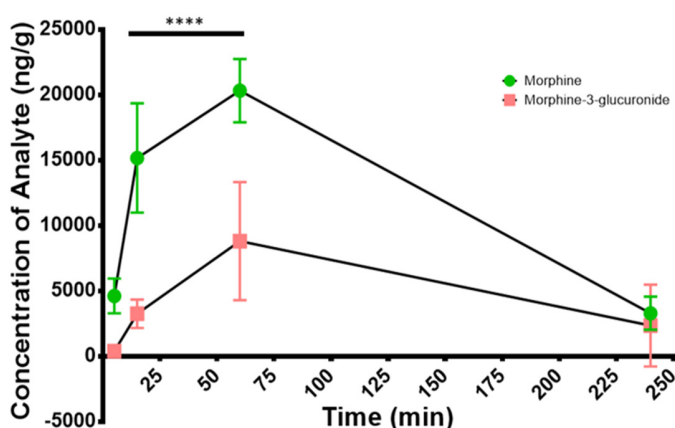In large intestine, average fentanyl AUC was 13798 ng/g*min, while average morphine AUC was 3842585 ng/g*min (p=0.0114). Fentanyl exhibited a t_{max} of 60 min, while morphine displayed a t_{max} of 240 min (**Figure S19a**). Due to a lack of norfentanyl data at 5, 15, and 60 min, statistical analysis could not be performed to compare fentanyl and norfentanyl concentrations, but norfentanyl concentration appears slightly higher relative to fentanyl (**Figure S19b**). No 4-ANPP was detected at any time point. No morphine-3-β-D-glucuronide was detected at any time point.

(a)

(b)

Figure S19. Concentration of Fentanyl, Morphine, and Select Metabolites in Large Intestine from 5-240 Min in Mice.
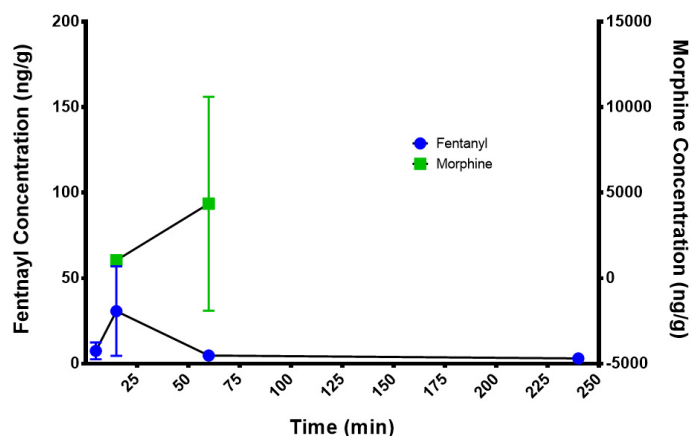
a) Average concentration of fentanyl and morphine in large intestine in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P = 0.0089; F(1, 6) = 14.52), but no significant effect of time (P = 0.1523; F (3, 18) = 1.985), or interaction of time and treatment (P = 0.1519; F(3, 18) = 1.988). Fentanyl AUC: 13798 ng/g*min; morphine AUC: 3842585 ng/g*min. *: p=0.0114

b) Average concentration of fentanyl and its metabolite, norfentanyl, in large intestine.

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In stomach, average fentanyl AUC was 19369 ng/g*min, while average morphine AUC was 3040544 ng/g*min ($p < 0.0001$). Both opioids displayed a t_{max} of 60 min (**Figure S20a**). Due to a lack of norfentanyl data at 5, 15, and 240 minutes, statistical analysis comparing fentanyl and norfentanyl concentrations could not be performed, but fentanyl levels appear higher at 60 min (**Figure S20b**). No 4-ANPP was detected at any time point. Morphine was detected at greater quantities than morphine-3-β-D-glucuronide at 15 min ($p < 0.0001$) and 60 min ($p < 0.0001$) (**Figure S20c**).

(a)

(b)

(c)

Figure S20. Concentration of Fentanyl, Morphine, and Select Metabolites in Stomach from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in stomach in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P<0.0001; $F(1, 6) = 146.5$), significant effect of time (P<0.0001; $F(3, 18) = 58.85$), and significant interaction of time and treatment (P = 0.0001; $F(3, 18) = 58.05$). Fentanyl AUC: 19369 ng/g*min; morphine AUC (original data): 3040544 ng/g*min. ****: p<0.0001

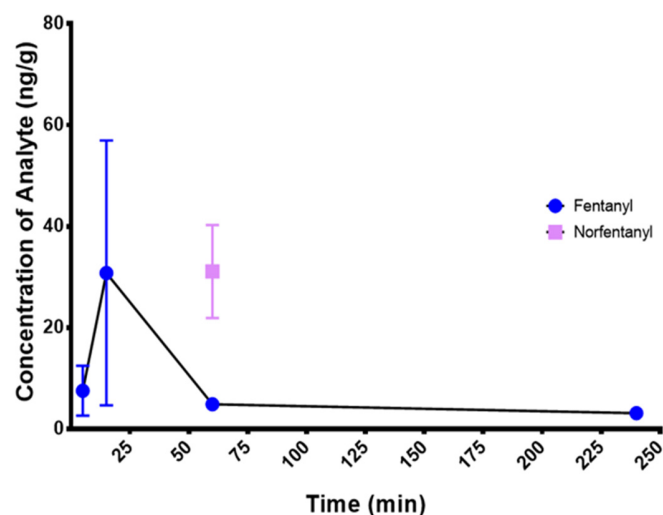b) Average concentration of fentanyl and its metabolite, norfentanyl, in stomach.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in stomach. Significant difference in analyte concentration (P=0.0004; $F (1, 6) = 48.91$), significant effect of time (P<0.0001; $F(3, 18) = 38.40$), and significant interaction of time and analyte concentration (P=0.0009; $F(3, 18) = 8.576$). ****:p<0.0001 (morphine vs morphine-3-β-D-glucuronide).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group
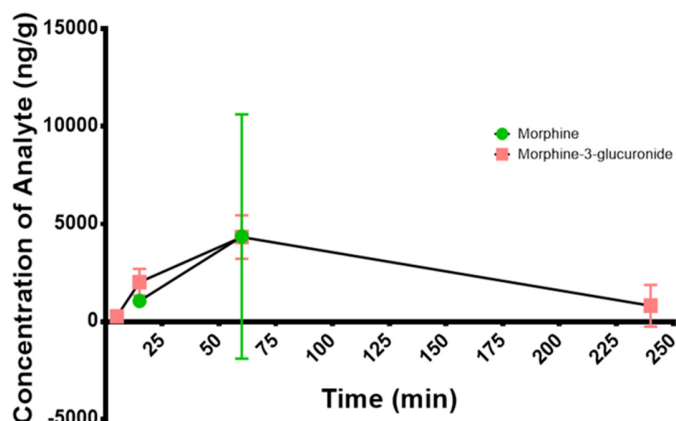
In muscle, average fentanyl AUC was 1467 ng/g*min, while average morphine AUC was 519773 ng/g*min (p=0.1903). Fentanyl displayed a t_{max} of 15 min, while morphine exhibited a t_{max} of 60 min (**Figure S21a**). Due to a lack of norfentanyl data at 5 min, 15 min, and 240 min, statistical analysis comparing fentanyl and norfentanyl could not be performed, but norfentanyl concentration appears higher at 60 min (**Figure S21b**). No 4-ANPP was detected at any timepoint. Due to lack of morphine data at 5 min and 240 min, statistical analysis comparing levels of morphine and morphine-3-β-D-glucuronide could not be performed, but measured concentrations appear similar at time points for which data was available (**Figure S21c**).

(a)

(b)

(c)

Figure S21. Concentration of Fentanyl, Morphine, and Select Metabolites in Muscle from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in muscle in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Fentanyl AUC: 1467 ng/g*min ; morphine AUC: 93818 ng/g*min. p=0.1903

b) Average concentration of fentanyl and its metabolite, norfentanyl, in muscle.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in muscle.

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

In fat, average fentanyl AUC was 28097 ng/g*min, while average morphine AUC was 328093 ng/g*min (p=0.0159). Both opioids exhibited a t_{max} of 60 min (**Figure S22a**). Due to a lack of norfentanyl data at 5 min and 15 min, statistical comparisons of fentanyl and norfentanyl levels could not be performed. However, fentanyl concentration appeared higher at 60 min and 240 min relative to its metabolite (**Figure S22b**). 4-ANPP was not detected at any time point. No significant differences were observed between morphine and morphine-3-β-D-glucuronide at any time point (**Figure S22c**).
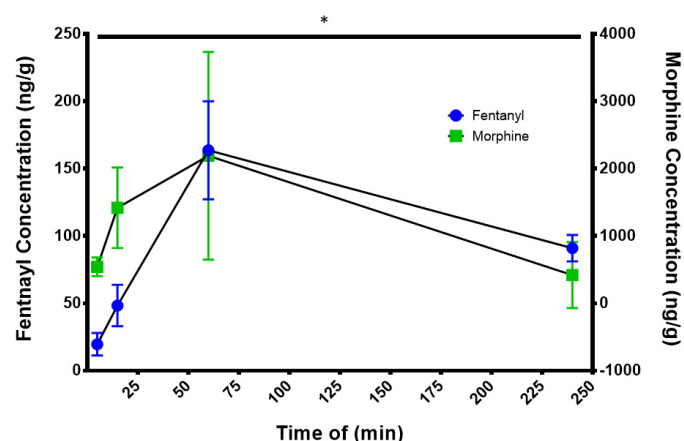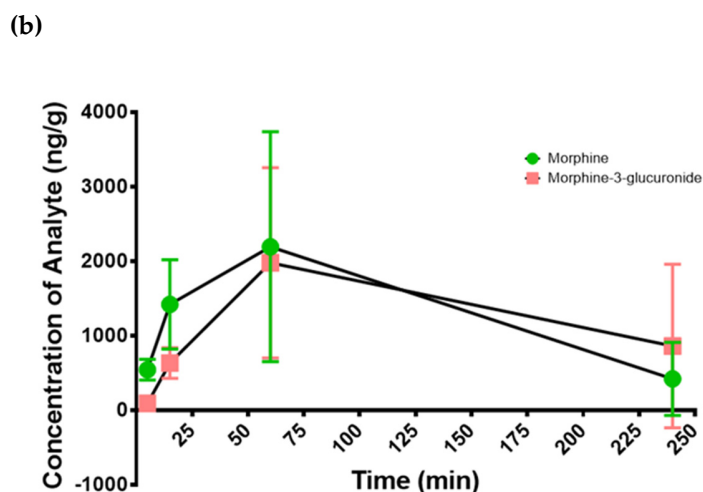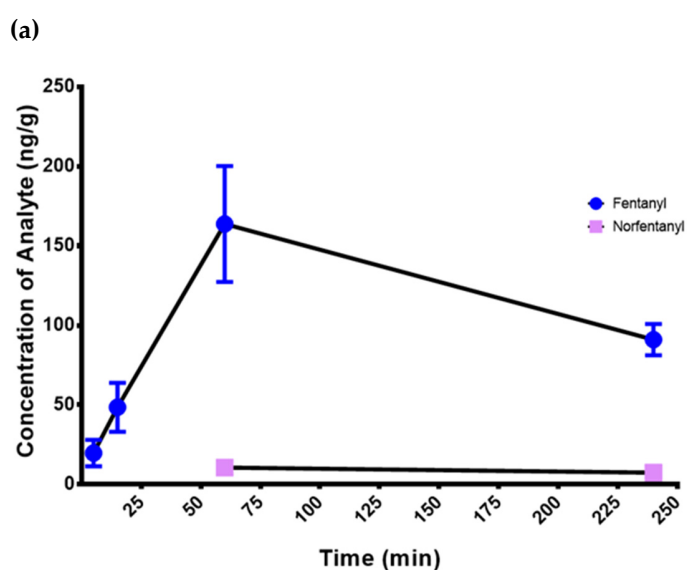
(a)

(b)

(c)

Figure S22. Concentration of Fentanyl, Morphine, and Select Metabolites in Fat from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in fat in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment ($P<0.0057$; $F(1, 6) = 17.66$), significant effect of time ($P<0.0142$; $F(3, 18) = 4.646$), and significant interaction of time and treatment ($P<0.0291$; $F(3, 18) = 3.774$). Fentanyl AUC: 28097 ng/g*min; morphine AUC: 328093 ng/g*min. *: p=0.0159.

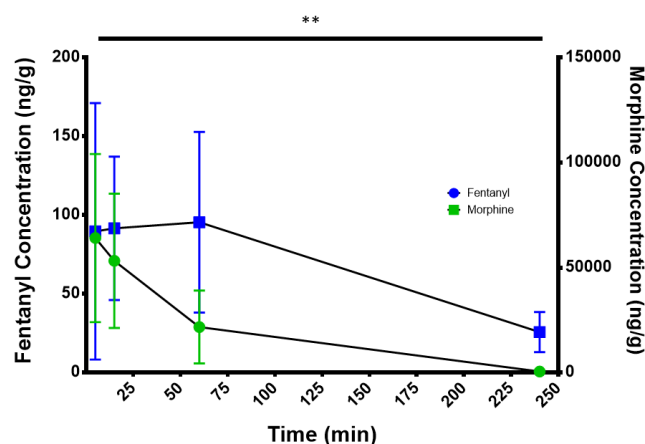b) Average concentration of fentanyl and its metabolite, norfentanyl, in fat.

c) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuruonide, in fat. No significant differences in analyte concentration ($P = 0.4752$; $F(1, 6) = 0.5799$) or significant interaction of time and analyte concentration ($P = 0.5157$; $F(3, 18) = 0.7889$, but significant effect of time ($P = 0.0027$; $F(3, 18) = 6.911$).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group
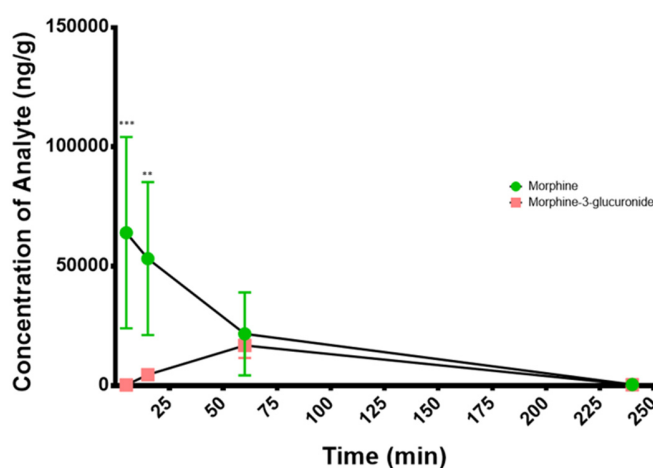
In skin, average fentanyl AUC was 16246 ng/g*min, while average morphine AUC was 4434730 ng/g*min (p=0.0017). Fentanyl exhibited a t_{max} of 60 min, while morphine displayed a t_{max} of 5 min (**Figure S23a**). No norfentanyl or 4-ANPP were detected at any time point. Morphine concentration was greater than morphine-3-β-D-glucuronide at 5 min (p<0.001) and 15 min (p<0.01) (**Figure S23b**).

(a)

(b)

Figure S23. Concentration of Fentanyl, Morphine, and Select Metabolites in Skin from 5-240 Min in Mice.

a) Average concentration of fentanyl and morphine in skin in mice injected with either 0.3 mg/kg fentanyl sc or 30 mg/kg morphine sc. Significant effect of treatment (P=0.0015; $F_{(1, 6)} = 30.29$), significant effect of time (P = 0.0165; $F_{(3, 18)} = 4.458$), and significant interaction of time and treatment (P = 0.01673; $F_{(3, 18)} = 4.443$). Fentanyl AUC: 16246 ng/g*min; morphine AUC: 4434730 ng/g*min. **: p=0.0017

b) Average concentration of morphine and its metabolite, morphine-3-β-D-glucuronide, in skin. Significant differences in analyte concentration (P = 0.0037; $F_{(1, 6)} = 21.14$), significant effect of time (P = 0.0197; $F_{(3, 18)} = 4.240$), and significant interaction of time and analyte concentration (P=0.0091; $F_{(3, 18)} = 5.220$). **: p<0.01 (morphine vs morphine-3-β-D-glucuronide); ***: p<0.001 (morphine vs morphine-3-β-D-glucuronide).

AUC compared by unpaired 2-tailed T-test. Analyte concentration data expressed as mean ± SD and analyzed by 2-way repeated-measures ANOVA, with Šídák's post hoc for multiple comparisons as needed. n = 4/group

The original AUC (area under the curve) values for fentanyl and morphine (individual subjects and treatment group averages) are displayed in **Table S21** and **Table S22**, respectively.

Table S21. Fentanyl AUC Values (Average and by Subject) in Blood and 12 Tissues

Tissue	AUC (ng/mL*min or ng/g*min)				
	Subject #1	Subject #2	Subject #3	Subject #4	Average
Blood	4719	3829	3640	2972	3790
Brain	14654	11862	12709	10957	12545
Liver	2258	1480	1884	1907	1882
Lung	34250	28658	42601	26605	33028
Heart	17391	20166	22227	15441	18806
Kidney	54849	52815	51797	46476	51484
Spleen	36738	36262	46554	38297	39463
Small intestine	11509	13561	20210	15181	15115
Large intestine	15885	10755	13756	14795	13798
Stomach	18580	18373	22782	17741	19369
Muscle	1547	524	2752	1045	1467
Fat	30551	24128	25136	32574	28097
Skin	17187	22002	17018	8778	16246

Table S22. Morphine AUC Values (Average and by Subject) in Blood and 12 Tissues

Tissue	AUC (ng/mL*min or ng/g*min)				
	Subject #1	Subject #2	Subject #3	Subject #4	Average
Blood	968106	1816140	1156793	792614	1183413
Brain	248860	393160	269930	267908	294964
Liver	214603	159278	172923	167490	178573
Lung	2496570	2985520	2410700	1677650	2392610
Heart	85361	65771	64372	126620	85531
Kidney	6773940	12431680	9560000	5247660	8503320
Spleen	3094169	4601706	3892459	2635961	3556074
Small intestine	2628740	2697500	1416700	1514920	2064465
Large intestine	2359010	6846860	3861810	2302660	3842585
Stomach	2858225	3390050	3282000	2631900	3040544
Muscle	281475	1566000	137800	93818	519773
Fat	587278	245980	302968	176148	328093
Skin	2880090	3785650	6737030	4336150	4434730