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Evidence for nonlinear accumulation of the ultrapotent fentanyl analog, carfentanil, after systemic administration to male rats

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Abstract

The current opioid overdose crisis is being exacerbated by illicitly manufactured fentanyl and its analogs. Carfentanil is a fentanyl analog that is 10,000-times more potent than morphine, but limited information is available about its pharmacology. The present study had two aims: 1) to validate a method for quantifying carfentanil and its metabolite norcarfentanil in small-volume samples, and 2) to use the method for examining pharmacodynamic-pharmacokinetic relationships in rats. The analytical method involved liquid-liquid extraction of plasma samples followed by quantitation of carfentanil and norcarfentanil using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The method was validated following SWGTOX guidelines, and both analytes displayed limits of detection and quantification at 7.5 and 15 pg/mL, respectively. Male Sprague-Dawley rats fitted with jugular catheters and temperature transponders received subcutaneous carfentanil (1, 3 and 10 µg/kg) or saline. Repeated blood specimens were obtained over 8 h, along with pharmacodynamic measures including core temperature and catalepsy scores. Carfentanil produced dose-related hypothermia and catalepsy that lasted up to 8 h. Carfentanil C_{max} occurred at 15 min whereas metabolite C_{max} was at 1-2 h. Concentrations of both analytes increased in a dose-related fashion, but area-under-the-curve values were much greater than predicted after 10 µg/kg. Plasma half-life for carfentanil increased at higher doses. Our findings reveal that carfentanil produces marked hypothermia and catalepsy, which is accompanied by nonlinear accumulation of the drug at high doses. We hypothesize that impaired clearance of carfentanil in humans could contribute to life-threatening effects of this ultrapotent opioid agonist.

1. Introduction

Since 2014, there has been an alarming rise in overdose deaths involving exposure to illicitly manufactured fentanyl and its various analogs (Jannetto et al., 2018; O'Donnell et al., 2017; Pichini et al., 2018). Carfentanil is a particularly dangerous fentanyl analog that is

encountered as a heroin adulterant and a constituent in counterfeit pain pills in the United States (US), Canada, and Europe (see Figure 1) (Drug Enforcement Administration, 2016; EMCDDA, 2017). In a recent study from the US, carfentanil was the most commonly detected fentanyl analog in post-mortem samples and associated with 1,236 opioid-related deaths (O'Donnell et al., 2018). In Europe, a total of 61 fatal carfentanil intoxications have been reported across eight countries, as well as 801 law enforcement seizures of the compound (EMCDDA, 2018). Carfentanil was first synthesized in the 1970s and displays analgesic potency that is 100- and 10,000-times greater than fentanyl and morphine, respectively (Armenian et al., 2018; Van Bever et al., 1976). The only approved use for carfentanil is in veterinary medicine as an immobilization agent for large animals at doses ranging from 5 to 20 µg/kg (George et al., 2010; WHO, 2017). Because carfentanil can be absorbed through the skin or inhaled, it is recommended that the opioid agonist naloxone is available when handling the substance (Swanson et al., 2017). In humans, the lethal dose is estimated to be as low as 20 µg (Casale et al., 2017), though this has not been empirically determined.

Surprisingly little information is available about the pharmacology and toxicology of carfentanil (see (Armenian et al., 2018; Prekupec et al., 2017). The drug binds to mu-opioid receptors with sub-nanomolar affinity (Baumann et al., 2018; Eriksson and Antoni, 2015; Titeler et al., 1989) and produces typical opioid effects in rodents, including antinociception, catalepsy, and respiratory depression (Bagley et al., 1991; Yong et al., 2014). One study in rats examined the pharmacodynamic effects of inhaled aerosolized carfentanil and noted loss of consciousness within 1 min of exposure, along with decreases in blood pressure, heart rate and core body temperature lasting for 24 h (Wong et al., 2017). After intramuscular (i.m.) administration in rats (250 µg/kg), the mean half-life ($t_{1/2}$) of carfentanil is reportedly 1.5 h (Yang et al., 2018). Due to the extremely high potency of carfentanil, concentrations in post-mortem blood and tissue are often in the low pg/mL range (Gergov et al., 2009; Tiscione and Alford, 2018). Thus, analytical methods for accurate detection of carfentanil require limits of quantification (LOQ) of 10–50 pg/mL (Shanks and Behonick, 2017). Recently, a number of sensitive analytical methods for the detection of carfentanil have been developed (Bergh et al., 2018; Cannaert et al., 2018; Hikin et al., 2018; Shanks and Behonick, 2017; Tiscione and Alford, 2018), but many forensic laboratories lack such methods and may fail to detect carfentanil in postmortem human samples (Elliott and Hernandez Lopez, 2018; Fomin et al., 2018; Shoff et al., 2017; Sofalvi et al., 2017; Swanson et al., 2017).

Few studies have investigated carfentanil metabolism and pharmacokinetics. An in vitro study using human hepatocytes identified the *N*-dealkylated metabolite norcarfentanil (Figure 1) as a major carfentanil metabolite (Feasel et al., 2016). Norcarfentanil has also been identified in blood and urine samples from human case work (Cannaert et al., 2018; Muller et al., 2018; Riches et al., 2012; Uddayasankar et al., 2018). In one recent case study, the $t_{1/2}$ values for carfentanil and norcarfentanil after recreational exposure were estimated to be 5.7 and 11.8 h, respectively. However, the kinetic profiles of both analytes were necessarily incomplete as maximal concentration (C_{max}) values were not captured (Uddayasankar et al., 2018). In an older study, intravenous administration of trace concentrations of ^{11}C carfentanil (0.02–0.04 µg/kg), used as a PET radioligand for the mu-

opioid receptor, yielded a $t_{1/2}$ of 42 min in healthy human controls, but terminal half life could not be calculated (Minkowski et al., 2012). To our knowledge, no analytical methods have been used to quantify carfentanil and its metabolites in rodent models given clinically-relevant carfentanil doses. Here, we report a validated analytical method using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for determination of carfentanil and norcarfentanil in small-volume plasma samples. Furthermore, we used the method to examine pharmacodynamic-pharmacokinetic relationships in rats receiving subcutaneous (s.c.) injections of carfentanil (1, 3, and 10 $\mu\text{g/kg}$). Our findings show that carfentanil produces marked hypothermia and catalepsy in rats, which is accompanied by impaired drug clearance at higher doses.

2. Materials and methods

2.1 Chemicals and reagents

Carfentanil and carfentanil- d_5 for analytical standards were acquired from Toronto Research chemicals (Toronto, Ontario, Canada). Norcarfentanil and norfentanyl- d_5 were acquired from Chiron AS (Trondheim, Norway). Chromasolv methanol (MeOH) of LC-MS grade was acquired from Honeywell Riedel-de Haën (Seelze, Germany). Carfentanil HCl used for animal experiments was obtained from NIDA Drug Supply Program (Rockville, MD, USA). A stock solution of 1 mg/mL carfentanil in sterile saline was prepared, and 100 μL aliquots were stored at -20°C . Ammonium bicarbonate and sodium metabisulfite were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate and formic acid (98%) were acquired from VWR International AS (Oslo, Norway). Nitric acid, ethyl acetate, sodium hydroxide and n-heptane were acquired from Merck (Darmstadt, Germany). Type 1 water (18.2 M Ω) purified with a Synthesis A 10 milli-Q system from Millipore (Billerica, MA, USA) was employed.

2.2 Analytical methods

Determination of carfentanil and norcarfentanil in rat plasma samples was performed using a previously described method (Bergh et al., 2018) with minor modifications. Stock solutions of carfentanil and norcarfentanil, and the internal standards (IS) carfentanil- d_5 and norfentanyl- d_5 , were prepared in MeOH and stored at -20°C . Working solutions for 7 calibrators and 5 quality control (QC) samples were prepared independently by dilution of stock solutions in MeOH. Blank blood plasma samples collected from drug-naïve control rats were pooled together and used for calibrators and QC samples. Calibrators (10-5000 pg/mL) and QC samples (7.5-4000 pg/mL) were prepared by fortifying 100 μL rat plasma with the working solutions.

Rat plasma samples (100 μL) were added to tubes containing MeOH and IS, then vortexed. Borate buffer (pH 11) was added followed by vortexing. A mixture of ethyl acetate/heptane was added to the tubes prior to 1 min of vortexing. The tubes were centrifuged, and supernatants were transferred to glass tubes containing nitric acid in MeOH, then evaporated to dryness under a stream of N_2 . The samples were reconstituted in mobile phase and centrifuged before the supernatants were transferred to autosampler vials for UHPLC-MS/MS analysis.

Analysis of rat plasma samples was performed using an Acquity UHPLC™ system (Waters, Milford, MA, USA) coupled to a Xevo-TQS triple quadrupole mass spectrometer with an electrospray ionization interface (Waters). Chromatographic separation of the analytes was performed using a Kinetex biphenyl column (Phenomenex, Verløse, Denmark) at 60 °C with a mobile phase consisting of 10 mM ammonium formate pH 3.1 (solvent A) and MeOH (solvent B) at a flow rate of 0.5 mL/min. The separation was carried out using a 9 min gradient profile. Data was acquired and processed using Masslynx™ 4.1 software (Waters). For more detailed information about the UHPLC-MS/MS analysis, see Bergh et al. (2018). The IS used for quantification of carfentanil and norcarfentanil were carfentanil-d₅ and norfentanyl-d₅, respectively. The MS/MS parameters used, and the retention time of the analytes and IS, are presented in Table 1.

2.3 Method validation

The method was validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines (Toxicology, 2013), with minor adjustments. The following validation parameters were examined: linearity, intermediate precision and bias, limit of detection (LOD), limit of quantification (LOQ), recovery, matrix effects (ME), matrix interferences, stability and carryover.

The linearity was assessed based on 8 assays of 7 calibrators prepared in pooled plasma from rats in the control group with one replicate per concentration level. Weighted calibration curves (1/x), excluding the origin, were constructed by plotting calibrator concentration against analyte/IS peak height ratio. Acceptable calibration curves had a correlation coefficient (R^2) 0.99 and residuals $\pm 20\%$.

Intermediate precision and accuracy were assessed based on 8 assays of 4 different QC sample concentrations prepared in both pooled plasma from the rats in the control group and in pooled Sprague Dawley rat plasma with added K₂EDTA (Tebu-Bio, Roskilde, Denmark) with one parallel pr. matrix. Accuracy given as bias was calculated as the percent deviation between the measured mean of the different QC samples and the respective nominal concentration. Precision was determined as the coefficient of variation (% CV). Intermediate precision and accuracy were calculated for all assays collectively and considered acceptable at a % CV and bias $\pm 20\%$.

LOD was assessed based on 3 assays of 5 different QC sample concentrations prepared in both pooled plasma from rats in the control group and pooled Sprague Dawley rat plasma with one parallel pr. matrix. LOD was defined as the lowest concentration that generated a signal-to-noise ratio (S/N) 3. LOQ was assessed based on 8 assays of 4 different QC sample concentrations prepared in both pooled plasma from rats in the control group and pooled Sprague Dawley rat plasma with one parallel pr. matrix. LOQ was defined as the lowest QC sample concentration where the intermediate precision and accuracy were $\pm 20\%$ and S/N 10 for the transition of quantification.

Recovery and ME were assessed by analyzing 3 sets of samples fortified with analytes at two concentration levels (25 and 4000 pg/mL). 10 blank plasma samples from 2 different sources (4 samples of pooled plasma from rats in the control group and 6 samples of

commercial pooled Sprague Dawley rat plasma) were fortified with analyte before extraction (set 1), or after extraction (set 2). In addition, 5 replicates of reconstitution solution were fortified with analyte (set 3). IS was added to all samples after the extraction. Recovery was calculated as the ratio of peak height of analyte added pre-extraction (set 1) to peak height of analyte added post-extraction (set 2). ME was calculated as the ratio of peak height of analyte added post-extraction (set 2) to peak height of analyte added to reconstitution solution (set 3), as described by Matuszewski et al. (2003). The ME was considered acceptable in the range of 80-120 %.

Matrix interferences were evaluated in the plasma samples taken from the rats prior to carfentanil exposure (N=17). Interference from the isotope labeled IS of carfentanil, carfentanil-d₅, which may contain traces of the unlabeled analyte, was evaluated by analysis of 8 blank samples fortified with IS.

The stability of the analytes in fortified pooled Sprague Dawley rat plasma and extracted rat plasma samples fortified pre-extraction were assessed in triplicates at two concentration levels (25 and 4000 pg/mL). The stability of fortified plasma samples was examined after storage for up to 2 months at -80 °C and after 2 freeze/thaw cycles. The stability of extracted samples was determined for up to 2 days in the autosampler at 10 °C. Samples were considered stable if the deviation from the initial concentration was ± 20 %.

Carry-over was examined by injecting an extracted rat plasma sample fortified with the highest calibrator (5000 pg/mL) followed by 3 samples of blank extracted matrix. Carry-over was present if the blank samples displayed a peak height >10 % of the peak height at LOQ.

2.4 Animals and surgery

Male Sprague-Dawley rats (300-400 g) purchased from Envigo (Frederick, MD, USA) were double-housed under conditions of controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($45\% \pm 5\%$), with ad libitum access to food and water. Lights were on between 7:00 AM and 7:00 PM. The Institutional Animal Care and Use Committee of the NIDA Intramural Research Program approved the animal experiments, and all procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Vivarium facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were designed to minimize the number of animals included in the study.

Rats were anesthetized with intraperitoneal (i.p.) ketamine and xylazine (75 and 5 mg/kg), and indwelling catheters were implanted into the right jugular vein. Catheters were constructed of Silastic (Dow Corning, Midland, MI, USA) and vinyl tubing as described previously (Concheiro et al., 2014). In brief, the proximal Silastic end of the catheter was advanced to the atrium while the distal vinyl end was exteriorized on the nape of the neck and plugged with a metal stylet. Immediately after catheter implantation, while the rats were still under anesthesia, temperature transponders (model IPTT-300; Bio Medic Data Systems, Seaford, DE, USA) were surgically implanted to allow noninvasive measurement of body temperature (Elmore and Baumann, 2018). The temperature transponder emits radio

frequency signals received by a compatible handheld reader system (DAS-7006/7r; Bio Medic Data Systems). The transponders are cylindrical in shape (14 x 2 mm) and were implanted s.c. along the midline of the back, posterior to the shoulder blades, via a prepackaged sterile guide needle delivery system. Rats were single housed postoperatively and given at least one week to recover from surgery.

2.5 Animal experiments

Rats were brought into the laboratory in their home cages on the day of an experiment and allowed 1 h to acclimate to the surroundings. Polyethylene extension tubes were attached to 1 mL tuberculin syringes and filled with sterile saline before being connected to the vinyl end of the catheters. Blood sampling, performed by an investigator remote from the animal, was facilitated by threading the extension tubes outside the cage. Catheters were flushed with 0.3 mL of 48 IU/mL heparin saline to facilitate blood withdrawal.

To prepare carfentanil for injection, a 100 μ L aliquot of 1 mg/mL carfentanil was thawed, then serially diluted in sterile saline to yield concentrations of 1, 3 and 10 μ g/mL. Groups of rats received s.c. injections of saline vehicle (control) or 1, 3, or 10 μ g/kg carfentanil on the lower back between the hips. Rats were randomly assigned to each dose group. Blood samples (300 μ L) were withdrawn via catheters immediately before and at 15, 30, 60, 120, 240, and 480 min after injection. Samples were collected into 1 mL tuberculin syringes and transferred to 1.5 mL plastic tubes containing 5 μ L of 1000 IU/mL heparin as an anticoagulant and 5 μ L of 250 mM sodium metabisulfite as a preservative. Blood was centrifuged for 10 min at 1000 g and 4°C. Plasma was decanted into cryovials and stored at -80°C until analysis. To maintain volume and osmotic homeostasis, rats received an equal volume of saline solution via the intravenous catheter after each blood withdrawal.

Body temperature and catalepsy scores were determined prior to each blood withdrawal. Rat behaviors were observed by an experienced rater for 1 min just prior to the measurement of body temperature via the handheld reader system. The behavioral rater was blind to treatment conditions. On each test day, one investigator prepared carfentanil solutions and administered the drug to rats, whereas another investigator performed the behavioral scoring without knowing the dose being administered to the animal. During the 1 min observation period, catalepsy behaviors were scored based on the following three overt symptoms: immobility, flattened body posture, and splayed limbs (Elmore and Baumann, 2018). At each time point, each symptom was scored as 1 = absent or 2 = present. For each animal, catalepsy scores at each time point were summed, yielding a minimum score of 3 and a maximum score of 6.

2.6 Plasma creatinine measures

Plasma creatinine concentration was determined by a colorimetric coupled enzyme reaction using a Creatinine Assay Kit (Sigma-Aldrich, Oslo, Norway). Absorbance was measured (570 nm) using an ELx808 Absorbance Microplate Reader (BIO-TEK Instruments Inc.).

2.7 Data analysis and statistics

Pharmacodynamic and pharmacokinetic data were statistically evaluated using GraphPad Prism version 7.04 (GraphPad Software, La Jolla, CA, USA). Time course data for body temperature, catalepsy scores and creatine concentrations were evaluated using two-way analysis of variance (dose x time) followed by Tukey's multiple comparison tests. Time-concentration time profiles for carfentanil and norcarfentanil were subjected to two-way analysis of variance (dose x time) followed by Tukey's multiple comparison tests. Formulas in Excel (Excel 2016, Microsoft, Redmond, WA, USA) were used to calculate pharmacokinetic constants for carfentanil and its metabolite, including C_{max} , area-under-the-curve (AUC), elimination constant (K_e) and plasma half-life ($t_{1/2}$). Pharmacokinetic constants for each analyte were compared by one-way analysis of variance (dose) followed by Tukey's tests to determine differences between dose groups. The observed AUC values, from 0 to 8 h post-injection, were compared to predicted AUC values which were calculated for the 3 and 10 $\mu\text{g/kg}$ doses by multiplying the observed value at 1 $\mu\text{g/kg}$ by 3 and 10, respectively. Predicted and observed values for carfentanil and norcarfentanil AUC were analyzed by two-way ANOVA (dose x condition) followed by Sidak's multiple comparison tests. Relationships between plasma concentrations of analytes and body temperature or catalepsy score were assessed using a Pearson's correlation analysis. Specifically, for each subject, the AUC value for each analyte was plotted with respect to the mean temperature or summed catalepsy score across all times points. $p < 0.05$ was used as the minimum threshold for statistical significance for all comparisons.

3. Results

3.1 Method validation

Figure 2 depicts representative MRM chromatograms for carfentanil and norcarfentanil in rat plasma samples analyzed with the presented UHPLC-MS/MS method. Carfentanil retention time was 5.4 min whereas metabolite retention time was 2.7 min. The LOD for carfentanil and norcarfentanil was 7.5 pg/mL while the LOQ for the compounds was 15 pg/mL. The calibration curves for both compounds were linear in the range of 10 to 5000 pg/mL with correlation coefficient (R^2) 0.99 and residuals $\pm 20\%$.

The validation data for carfentanil and norcarfentanil are summarized in Tables 2 and 3. The intermediate precision and accuracy for carfentanil and norcarfentanil were acceptable for all four QC concentrations (15, 25, 500 and 4000 pg/mL) with a % CV 15% and bias 7.6%. No matrix interferences were observed for carfentanil in the plasma samples. In the norcarfentanil chromatograms, peaks were observed, but the height was $< 20\%$ of the peak height at LOQ and therefore not likely to interfere with analyte quantification. Recovery was high for carfentanil (88 - 91%) and norcarfentanil (81 - 84%), and the two compounds did not display ME (89 - 103%). Carry-over was not observed for either analyte. Stability studies were performed in fortified rat plasma samples and extracted plasma samples fortified pre-extraction (Table 3). Carfentanil and norcarfentanil were stable in fortified plasma for two freeze/thaw cycles and for up to 2 months storage at -80°C . Both compounds were stable in extracted plasma samples stored in the autosampler (10°C) for 48 h.

3.2 Pharmacodynamic effects

The data in Figure 3 depict the time-course effects of s.c. carfentanil administration on core body temperature and catalepsy in male rats. Body temperature was significantly affected by dose ($F[3,133]=34.47$, $p < 0.0001$) and time ($F[6,133]=15.47$, $p < 0.0001$), with a significant dose x time interaction ($F[18,133]=6.02$, $p < 0.0001$). Carfentanil induced a dose-related decrease in temperature when compared to saline-treated controls. No significant temperature change was found after 1 $\mu\text{g/kg}$, but a significant hypothermic response was observed after 3 $\mu\text{g/kg}$ that reached a nadir of 2.4 °C compared to control at 2 h post-injection. The 10 $\mu\text{g/kg}$ dose of carfentanil produced significant hypothermia that reached a nadir of 3.7 °C at 2 h post-injection. Temperature measures returned to control values by 4 h after 3 $\mu\text{g/kg}$ and by 8 h after 10 $\mu\text{g/kg}$ carfentanil.

Catalepsy score was significantly affected by carfentanil dose ($F[3,133]=109.71$, $p < 0.0001$) and time ($F[6,133]=35.47$, $p < 0.0001$), with a significant dose x time interaction ($F[18,133]=8.57$, $p < 0.0001$). Carfentanil produced a dose-related increase in catalepsy when compared to saline-treated controls. This effect was characterized by immobility, flattened body posture and splayed hind limbs. Other effects of the drug included exophthalmos and shallow labored breathing, but these endpoints were not systematically evaluated. The 1 $\mu\text{g/kg}$ dose of carfentanil increased catalepsy score only at 15 min post-injection, whereas the 3 $\mu\text{g/kg}$ dose increased catalepsy for 2 h post-injection. At the 10 $\mu\text{g/kg}$ dose, carfentanil increased catalepsy scores for the entire 8 h session.

3.3 Plasma pharmacokinetics

Figure 4 illustrates the time-concentration profiles for plasma carfentanil and norcarfentanil after s.c. administration of carfentanil to male rats. The time-concentration profiles for carfentanil were significantly affected by dose ($F[2,84]=118.2$, $p < 0.0001$) and time ($F[5,84]=37.84$, $p < 0.0001$), with plasma concentrations rising as dose increased. Post hoc tests revealed that plasma concentrations of carfentanil were significantly higher after 10 $\mu\text{g/kg}$ when compared to 1 $\mu\text{g/kg}$ for 2 h post-injection, whereas there was no significant difference between 3 $\mu\text{g/kg}$ and 1 $\mu\text{g/kg}$ doses at any point. Pharmacokinetic constants are reported in Table 4. The C_{max} after carfentanil injection was significantly altered by the dose administered ($F[2,14]=60.93$, $p < 0.001$), as was AUC ($F[2,14]=13.57$, $p < 0.001$), K_e ($F[2,14]=9.091$, $p < 0.003$), and $t_{1/2}$ ($F[2,14]=6.17$, $p < 0.01$). Carfentanil T_{max} occurred at 15 min post-injection for all doses and was not affected by the dose administered. Post hoc tests showed that C_{max} and AUC values after administration of a 10 $\mu\text{g/kg}$ dose were significantly greater than those observed after 1 and 3 $\mu\text{g/kg}$ doses, while K_e and $t_{1/2}$ values were significantly different after 10 $\mu\text{g/kg}$ when compared to 1 $\mu\text{g/kg}$.

The time-concentration profiles for plasma norcarfentanil were also significantly affected by carfentanil dose ($F[2,84]=76.44$, $p < 0.0001$) and time post-injection ($F[5,84]=16.35$, $p < 0.0001$), with concentrations rising as dose increased (Figure 4). The plasma concentrations of norcarfentanil were significantly higher after 3 $\mu\text{g/kg}$ carfentanil when compared to those observed after 1 $\mu\text{g/kg}$ at the 1 h time point, while the norcarfentanil concentrations after 10 $\mu\text{g/kg}$ were significantly higher than those after 1 $\mu\text{g/kg}$ from 1 to 8 h post-injection. Pharmacokinetic constants for norcarfentanil are reported in Table 4.

Norcarfentanil $C_{\max}$ was significantly altered by the dose administered ($F[2,14]=19.89$, $p<0.0001$), as was AUC ($F[2,14]=41.14$, $p<0.001$). Norcarfentanil $T_{\max}$ occurred between 1-2 h and was not affected by the dose administered. Post hoc tests showed that $C_{\max}$ and AUC values after administration of a 10 $\mu\text{g/kg}$ dose were significantly greater than those observed after 1 and 3 $\mu\text{g/kg}$ doses.

As noted above, $t_{1/2}$ values for carfentanil were increased at the 10 $\mu\text{g/kg}$ dose, suggesting the possibility of nonlinear accumulation of the drug due to impaired clearance at the high dose. To examine the possibility of nonlinear pharmacokinetics for carfentanil and norcarfentanil, we compared the observed AUC values at 3 and 10 $\mu\text{g/kg}$ to the predicted values, which were determined by multiplying the observed values at 1 $\mu\text{g/kg}$ by a factor of 3 and 10, respectively. Figure 5 depicts the observed versus predicted AUC values for carfentanil and norcarfentanil at the doses of carfentanil administered. Two-way ANOVA demonstrated that observed versus predicted AUC values for carfentanil differed significantly ($F[1,28]=8.04$, $p<0.01$), with observed values being greater than predicted at 10 $\mu\text{g/kg}$. Similarly, observed AUC for norcarfentanil differed significantly from predicted AUC ($F[1,28]=16.30$, $p<0.001$), with observed AUC at 10 $\mu\text{g/kg}$ being greater than predicted. The findings presented in Figure 5 indicate impaired clearance of both carfentanil and norcarfentanil at the high drug dose.

3.4 Assessment of kidney function

Because drug clearance is largely mediated by the kidneys, we examined plasma concentrations of creatinine in carfentanil-treated rats as a gauge of renal function. Creatinine was assayed only in samples from the drug-treated groups, as there was insufficient plasma for analysis in the vehicle-treated group. The data in Table 5 demonstrate that plasma creatinine concentrations were significantly altered by carfentanil dose ($F[2,84]=6.54$, $p<0.003$) and time after dosing ($F[5,84]=7.68$, $p<0.001$). Plasma creatinine remained stable after the 1 $\mu\text{g/kg}$ dose of carfentanil but rose slowly over time in the 3 and 10 $\mu\text{g/kg}$ groups. Post hoc tests revealed that plasma creatinine was significantly elevated at 8 h in the 3 and 10 $\mu\text{g/kg}$ dose groups when compared to the 1 $\mu\text{g/kg}$ dose group.

3.5 Correlation analyses

Since the pharmacodynamic and pharmacokinetic endpoints were measured from the same rats, the relationships between temperature and catalepsy could be related to plasma analyte concentrations. Here we plotted the mean temperature and catalepsy scores for each rat against the AUC values from those same subjects. Figure 6 shows that carfentanil AUC is significantly correlated with mean body temperature and summed catalepsy scores. Body temperature was negatively correlated with carfentanil AUC (Pearson's $r = -0.751$, $p < 0.001$) whereas catalepsy was positively correlated with carfentanil AUC (Pearson's $r = 0.784$, $p < 0.001$). Similar findings were observed when relating norcarfentanil AUC to body temperature (Pearson's $r = -0.712$, $p < 0.001$) and catalepsy scores (Pearson's $r = 0.832$, $p < 0.001$).

4. Discussion

The global rise in opioid overdose fatalities is being driven by the presence of fentanyl and its various analogs. These compounds are encountered as heroin adulterants, standalone products, and ingredients in counterfeit pain pills (Jannetto et al., 2018; O'Donnell et al., 2017; Pichini et al., 2018). Most users are unaware of their exposure to fentanyl and its analogs. Carfentanil is an example of an ultrapotent fentanyl analog that is associated with many analytically-confirmed fatalities (Elliott and Hernandez Lopez, 2018; Papsun et al., 2017; Shanks and Behonick, 2017; Swanson et al., 2017). A major challenge for effective carfentanil surveillance is the extraordinarily low concentrations of the drug in clinical case work and post-mortem tissue samples (e.g., see (Shoff et al., 2017)). As a result, scant information is available about carfentanil pharmacokinetics and metabolism in humans or laboratory animals.

Here, we report a sensitive analytical method for the quantitation of carfentanil and norcarfentanil that can be used to examine pharmacodynamic-pharmacokinetic relationships in rats. We demonstrate that carfentanil induces marked hypothermia and catalepsy at s.c. doses from 3-10 µg/kg. Carfentanil $C_{\max}$ values in rat plasma ranged from 613 pg/mL at 1 µg/kg to 7682 pg/mL at 10 µg/kg, similar to the circulating concentrations measured in human performance case work (Papsun et al., 2017; Sofalvi et al., 2017; Tiscione and Alford, 2018). Plasma concentrations of carfentanil and norcarfentanil increased as the administered dose of carfentanil increased, but AUC values for both analytes after 10 µg/kg were significantly higher than predicted by simple linear kinetics. Carfentanil $t_{1/2}$ was extended with increasing doses, further supporting the notion of impaired clearance at higher doses. If the nonlinear accumulation of carfentanil observed in rats also occurs in humans, this phenomenon could contribute significantly to life-threatening adverse effects and resistance to naloxone rescue.

Preclinical studies show that carfentanil displays sub-nanomolar affinity for mu-opioid receptors (Baumann et al., 2018; Eriksson and Antoni, 2015; Titeler et al., 1989) and produces typical opioid effects such as antinociception, catalepsy and respiratory depression at very low doses (Bagley et al., 1991; Van Bever et al., 1976; Yong et al., 2014). Here we used radiotelemetry to demonstrate that carfentanil induces robust hypothermia in male Sprague-Dawley rats given s.c. doses of 3 and 10 µg/kg. Our temperature results in rats are consistent with those of Wong et al. (2017) who exposed mice to aerosolized carfentanil and found suppression of body temperature for up to 24 h post-treatment. It is well established that mu-opioid receptor agonists cause differential effects on core temperature in rats, with low doses producing hyperthermia and high doses producing hypothermia (Adler et al., 1988; Rawls and Benamar, 2011). Early work by Geller et al. (1983) showed that s.c. morphine increases body temperature in male Sprague-Dawley rats at doses between 4-16 mg/kg, whereas the drug decreases temperature at doses greater than 16 mg/kg. When compared to our study, Geller et al. found decreases in rat body temperature after 32 mg/kg morphine that mirrored the effects we observed after 3 µg/kg carfentanil. Taken together, the results indicate that carfentanil is about 10,000-fold more potent than morphine in its ability to produce hypothermia in the rat. The temperature findings agree with prior work showing

that carfentanil is 10,000-fold more potent than morphine as an analgesia agent (Bagley et al., 1991; Van Bever et al., 1976).

The administration of mu-opioid receptor agonists like morphine and fentanyl causes catalepsy in rats that is characterized by immobility and muscle rigidity (Chen et al., 1996; Ling and Pasternak, 1982; Pasternak et al., 1983). Indeed, carfentanil is approved for veterinary use as an immobilization agent for large animals, at doses ranging from 5 to 20 $\mu\text{g/kg}$ (George et al., 2010; WHO, 2017). In most rodent studies, catalepsy is measured by determining latency in the bar test or disruption of the righting reflex (Pasternak et al., 1983; Yong et al., 2014). Here we used a behavioral scoring method to assess catalepsy without physically disturbing the rats during the blood sampling procedures. Catalepsy was scored based on immobility, flattened body posture and splayed limbs. Using this simple scoring method, we show that catalepsy lasts up to 8 h after a 10 $\mu\text{g/kg}$ dose of carfentanil. Future investigations should examine the precise receptor mechanisms responsible for cataleptic and other adverse effects of carfentanil, as such studies could lead to the discovery of novel antagonists for opioid overdose rescue.

Here, we validated a UHPLC-MS/MS method that allows for quantitation of carfentanil and norcarfentanil in small-volume rat plasma samples, even after a low dose of 1 $\mu\text{g/kg}$ carfentanil. The method displays a LOQ of 15 pg/mL for carfentanil and norcarfentanil, which is comparable or superior to other methods reported in the literature (Bergh et al., 2018; Cannaert et al., 2018; Elliott and Hernandez Lopez, 2018; Fomin et al., 2018; Hikin et al., 2018; Shanks and Behonick, 2017; Shoff et al., 2017; Swanson et al., 2017; Tiscione and Alford, 2018). Additionally, our method is characterized by acceptable precision and accuracy, coupled with minimal matrix interference. Much of the information about carfentanil concentrations in human blood and tissues comes from post-mortem case work, but recent studies have reported carfentanil concentrations in blood from human drugged-driving cases (Papsun et al., 2017; Sofalvi et al., 2017; Tiscione and Alford, 2018). Results from these cases show circulating carfentanil levels ranging from 100 to 1400 pg/mL , and most of the individuals had other drugs of abuse or alcohol present. The blood concentrations of carfentanil measured in human case work overlap with the plasma concentrations of the drug we measured in rats given s.c. injections of 1 and 3 $\mu\text{g/kg}$ doses (see Figure 4), suggesting our preclinical rat model has translational value.

Few investigations have examined pharmacokinetics of carfentanil or its metabolites in laboratory animals or humans. In the only rat study available, Yang et al. (2018) reported a carfentanil $t_{1/2}$ of 1.5 h after i.m. administration of 250 $\mu\text{g/kg}$. We found much lower $t_{1/2}$ values in rats, ranging from 35 min after 1 $\mu\text{g/kg}$ to 64 min after 10 $\mu\text{g/kg}$. An obvious difference between the experiments of Yang et al. and the present work is the administration of a 25-fold higher dose in the former study. Other investigators have reported carfentanil $t_{1/2}$ values of 5.5 h in goats given 40 $\mu\text{g/kg}$ (Mutlow et al., 2004) and 3.7 h in elands given 16.9 $\mu\text{g/kg}$ (Cole et al., 2006). It is noteworthy that carfentanil C_{max} values reported in goats and elands immobilized by carfentanil administration ranged from 9000 to 13,000 pg/mL , and these values are close to the 7800 pg/mL that we measured in rats immobilized by 10 $\mu\text{g/kg}$ of carfentanil. Studies aimed at determining the $t_{1/2}$ for carfentanil in humans are limited by obvious logistical and ethical constraints. In a recent clinical overdose case, the

$t_{1/2}$ values for carfentanil and norcarfentanil were estimated to be 5.7 and 11.8 h, respectively. However, the kinetic profiles of both analytes were incomplete as $C_{\max}$ values were not obtained (Uddayasankar et al., 2018). We observed norcarfentanil concentrations in rats that were about 10-fold lower than those of the parent compound across all doses, and this result agrees with human studies measuring both analytes in carfentanil overdose cases (Cannaert et al., 2018; Muller et al., 2018; Riches et al., 2012; Uddayasankar et al., 2018).

Perhaps the most important observation from the present study is the nonlinear accumulation of carfentanil and norcarfentanil in the bloodstream after administration of increasing carfentanil doses. The occurrence of nonlinear kinetics is supported by at least three pieces of evidence. First, the observed AUC values for both analytes after the 10 $\mu\text{g/kg}$ dose of carfentanil were significantly greater than the predicted AUC values based on simple linear kinetics. Second, the $t_{1/2}$ values for carfentanil and norcarfentanil increased as the dose increased, consistent with reduced clearance of both compounds from the circulation after higher doses. Finally, delayed elevations in plasma creatinine concentrations after 3 and 10 $\mu\text{g/kg}$ carfentanil suggest impaired renal function which could contribute to reduced drug clearance. The time course and extent of kidney dysfunction after carfentanil administration are not known. Prior clinical data show that high doses of morphine induce a marked but transient reduction in urinary flow rate and a robust decrease in glomerular filtration rate (Mercadante and Arcuri, 2004). Both fentanyl and carfentanil induce substantial decreases in mean arterial pressure in rodents (Wong et al., 2017; Yadav et al., 2018), and it is tempting to speculate this effect could contribute to reduction in urine output. Regardless of the mechanisms involved, we hypothesize that nonlinear accumulation of carfentanil in humans could exacerbate the inherent dangers of this ultrapotent opioid agonist. Future studies should examine the effects of dose and route on pharmacokinetics of carfentanil and other fentanyl analogs in laboratory animal models.

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Figure 1.
Chemical structures of fentanyl, carfentanil and norcarfentanil.

Figure 2. MRM chromatograms of a rat plasma sample obtained 60 min after injection of 3 µg/kg carfentanil analyzed with the presented UHPLC-MS/MS method.

Figure 3.

Dose-response effects of s.c. administered carfentanil on body temperatures and catalepsy scores in male rats. Rats fitted with indwelling jugular catheters and temperature responders received s.c. vehicle (1 mL/kg) or carfentanil (1, 3, or 10 µg/kg) at time zero. Temperature and behavior were measured prior to blood sampling at 15, 30, 60, 120, 240, and 480 min after injection. Data are expressed as the mean $\pm$ SEM for N=5-6 rats/group. Filled symbols indicate significant differences compared with vehicle control at a given time point (Tukey's multiple comparison test, $p < 0.05$).

Figure 4.

Time-concentration profiles for carfentanil and norcarfentanil in rats receiving 1, 3, or 10 µg/kg s.c. carfentanil. Rats fitted with indwelling jugular catheters and temperature transponders received carfentanil s.c. at time zero. Blood samples were withdrawn via the catheters immediately prior to and 15, 30, 60, 120, 240, and 480 min after carfentanil injection. Plasma samples were assayed for analytes using UHPLC-MS/MS. Data are expressed as the mean $\pm$ SEM for N=5-6 rats/group. Filled symbols indicate significant differences compared to the low-dose group (1 µg/kg) at a given time point (Tukey's multiple comparison test, $p < 0.05$).

Figure 5.

Comparison of observed versus predicted area-under-the-curve (AUC) values for carfentanil and norcarfentanil after s.c. administration of carfentanil in rats. Observed AUC values were obtained from time-concentration profiles depicted in Figure 4, whereas predicted values at 3 and 10 µg/kg doses were calculated by multiplying the observed AUC values at 1 µg/kg by 3 and 10, respectively. Data are expressed as the mean $\pm$ SEM for N=5-6 rats/group. Filled bars indicate significant differences when compared to the predicted AUC value at a given dose (Sidak's multiple comparison test, $p < 0.05$).

Figure 6. Correlations between AUC values for carfentanil and nor-carfentanil versus body temperatures and catalepsy scores. Raw data from Figures 3 and 4 were used to construct the correlation matrices which plot mean temperature and summed catalepsy scores for each rat versus corresponding AUC values for each analyte in the same subjects over the 8 h session. Pearson's r and p values are shown.

Table 1.

MRM transitions, cone voltages, collision energies and retention times.

Analyte	MRM Transitions	MS/MS parameters		Retention time (RT) ^a	
		Cone voltage (V)	Collision energy (eV)	RT	CV (%)
Carfentanil	395.1 > 335.2	12	16	5.4	0.7
	395.1 > 113.1	12	28		
Norcarfentanil	291.2 > 142.3	8	14	2.7	0.4
	291.2 > 113.3	8	26		
Carfentanil-d ₅	400.2 > 330.2	12	16	5.4	0.2
	400.2 > 133.1	12	28		
Norfentanyl-d ₅	238.5 > 84.1	18	16	2.5	0.7
	238.5 > 155.1	18	16		

^aRT was calculated based on 8 assays of calibrators and QC samples.

Table 2.

Intermediate precision and accuracy, matrix effects and recovery.

Analyte	Nominal Conc.	Intermediate precision and accuracy		Matrix Effects (ME)			Recovery	
				ME (%)	CV (%)	ME corr (%) ^a	%	CV (%)
Carfentanil	15	9.5	-7.5					
	25	7.9	-7.6	97	13	107	91	11
	501	7.6	-3.1					
	4008	6.6	3.2	103	2.3	101	88	12
Norcarfentanil	15	15	4.4					
	25	8.3	3.8	89	5.8	91	84	10
	501	9.1	-2.4					
	4008	6.4	-0.6	98	2.8	97	81	5.1

^a ME corrected with Internal Standard

Table 3.

Stability data for carfentanil and norcarfentanil in extracted samples stored at 10 °C and in plasma stored at –80 °C.

Analyte	Nominal Conc. (pg/mL)	Stability (%) ^a					
		Extracted samples stored at 10 °C			Plasma samples stored at –80 °C		
		24 h	48 h	24 h	1 month	2 months	2 freeze-thaw cycles
Carfentanil	25	7.9 (2.8)	–7.7 (7.1)	6.3 (6.1)	–7.2 (5.3)	–3.4 (1.9)	0.7 (6.7)
	4008	–8.8 (3.2)	–9.9 (3.4)	–14 (4.3)	–20 (5.8)	–18 (10)	8.2 (3.7)
Norcarfentanil	25	1.7 (8.2)	11 (5.1)	8.7 (11)	–5.8 (7.0)	–4.9 (3.0)	–3.0 (9.3)
	4008	–4.6 (1.0)	1.1 (3.1)	–8.2 (3.2)	–13 (2.1)	–16 (11)	5.0 (1.5)

^a% CV in parentheses

Table 4.

Pharmacokinetic constants for carfentanil and norcarfentanil after s.c. administration of 1, 3, or 10 µg/kg carfentanil in male rats.

Analyte	Carfentanil dose (µg/kg)	C _{max} (pg/mL)	AUC _{0-8 h} (min * ng/mL)	K _e (min ⁻¹)	t _{1/2} (min)
Carfentanil	1 (N=6)	613 ± 49	29 ± 3	0.020 ± 0.002	35.4 ± 2.5
	3 (N=6)	1902 ± 418	148 ± 25	0.013 ± 0.001 *	55.1 ± 6.3
	10 (N=5)	7862 ± 804 *#	696 ± 184 *#	0.012 ± 0.002 *	64.4 ± 8.4 *
Norcarfentanil	1 (N=6)	40 ± 4	7 ± 1	0.004 ± 0.001	176 ± 10
	3 (N=6)	211 ± 38	38 ± 5	0.004 ± 0.001	350 ± 215
	10 (N=5)	601 ± 116 *#	128 ± 18 *#	0.002 ± 0.001	537 ± 200

Data are mean ± SEM for group size given.

Abbreviations are: maximal concentration (C_{max}), area-under-the-curve from 0-8 h (AUC_{0-8 h}); elimination rate constant (K_e), and half-life (t_{1/2})

* = significant difference compared to 1 µg/kg dose (Tukey's p<0.05)

= significant difference compared to 3 µg/kg dose (Tukey's, p<0.05)

Table 5.

Plasma creatinine concentrations after s.c. administration of 1, 3, or 10 µg/kg carfentanil in male rats.

Carfentanil Dose	Plasma Creatinine (µmol/L) ^a					
	0	30 min	1 h	2 h	4 h	8 h
1 µg/kg	72 ± 8	68 ± 8	78 ± 6	87 ± 7	93 ± 11	80 ± 9
3 µg/kg	78 ± 7	69 ± 6	79 ± 8	106 ± 13	125 ± 17	137 ± 44 *
10 µg/kg	69 ± 13	86 ± 9	94 ± 12	107 ± 17	147 ± 23	192 ± 31 *

^aCreatinine was measured via enzymatic reaction as described in Materials and Methods.

* = significant difference compared to 1 µg/kg dose (Tukey's $p < 0.05$)