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Carfentanil toxicity in the African green monkey: Therapeutic efficacy of naloxone

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ABSTRACT

Carfentanil is an ultra-potent opioid with an analgesic potency 10,000 times that of morphine but has received little scientific investigation. Three experiments were conducted to evaluate the toxicity of carfentanil and the efficacy of naloxone in adult male African green monkeys. The first experiment determined the ED_{50} (found to be 0.71 μg/kg) of subcutaneous carfentanil for inducing bradypnea and/or loss of posture. Experiment 2 attempted to establish the ED_{50} of naloxone for rapidly reversing the bradypnea/loss of posture induced by carfentanil (1.15 μg/kg). Experiment 3 evaluated the effects of carfentanil (0.575 μg/kg) alone, the safety of naloxone (71 − 2841 μg/kg), and the efficacy of naloxone (71 − 710 μg/kg) administration at two time points following carfentanil (1.15 μg/kg) on operant choice reaction time. Collectively, these experiments characterized the temporal progression of carfentanil-induced toxic signs, determined the range of naloxone doses that restored respiratory and gross behavioral function, and determined the time course and range of naloxone doses that partially or completely reversed the effects of carfentanil on operant choice reaction time performance in African green monkeys. These results have practical relevance for the selection of opioid antagonists, initial doses, and expected functional outcomes following treatment of synthetic opioid overdose in a variety of operational/emergency response contexts.

1. Introduction

Carfentanil is an ultra-potent opioid with a clinical potency up to 10,000 times that of morphine and 100 times of fentanyl (George et al., 2010; Lust et al., 2011), and has received little scientific study in common laboratory animal species/models. However, carfentanil has been implicated in an increasing number of opioid overdose deaths (O'Donnell et al., 2018) due to its high potency and relative ease and cost of illicit manufacture (World Health Organization, 2017). The most prevalent reports of carfentanil in the scientific literature are for the immobilization of wildlife and as a tool for neuroimaging studies investigating μ-opiate receptor binding and occupancy in humans (Eriksson and Antoni, 2015; Ingman et al., 2005; Lian et al., 2016). Obviously, while helpful, these studies do not fulfill the need for controlled studies across a range of relevant doses and resulting intoxication. A recent study from this institute (Wong et al., 2017) investigated the effects of aerosolized carfentanil on physiological function in mice and provided a preliminary evaluation of naloxone efficacy in reversing carfentanil-induced respiratory depression. Another recent rodent study (Yong et al., 2014) investigated the ability of nalmefene to reverse the respiratory depression and suppression of righting reflex induced by carfentanil in rats. An older study (Port et al., 1984) evaluated the analgesia and anesthesia produced by intravenous (IV) carfentanil in dogs and female rhesus monkeys and attempted to determine an anesthetic ED_{50} for IV carfentanil in both species. The investigators evaluated two monkeys at each of four carfentanil doses (0.1, 0.3, 0.6, and 1.0 μg/kg). Monkeys administered carfentanil at doses greater than or equal to 0.6 μg/kg experienced respiratory depression and required reversal with naloxone. The Port et al. (1984) study provided an approximate starting dose for the present set of studies, which were undertaken to advance our understanding of carfentanil toxicity via subcutaneous injection in a nonhuman primate model. The subcutaneous injection of carfentanil was selected because it provides a controlled route of administration and drug absorption, is safely accomplished by investigative personnel, and is commonly used in a variety of laboratory species. The African green monkey was selected for use in the present studies because of its phylogenic similarity to other common laboratory primates as well as to humans, and because African green monkeys offer several practical advantages over macaque species, including availability, cost, temperament, and reduced likelihood of fatal disease transmission to humans (cf. Myers and Hamilton, 2011).

Behavioral pharmacology/toxicology is well-suited to establishing

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the toxicity of chemical threats and the safety and efficacy of medical countermeasures against chemical threats. The control of operant behavior in laboratory settings for the evaluation of the effects of drugs/chemicals has proven quite useful, and the behavioral pharmacology and behavioral toxicology disciplines are well recognized (Barrett, 2002; Dews, 1956; Kelleher and Morse, 1968; Rice, 1988; Weiss and Laties, 1969). Operant behavioral assessments are considered apical tests of nervous system function because they require the *integrated function* of multiple organ systems (Weiss and Cory-Slechta, 2001), including the broader peripheral and central nervous systems. The use of behavior to assess the safety, efficacy, and toxicity of a wide variety of chemicals and countermeasures relies on the sensitivity of operant behavioral performances to disruption by a broad array of chemicals. Upon determining an effective dose of a medical countermeasure, behavioral safety assessments form the core of advanced toxicity testing. Even subtle changes across time can be discerned using well implemented operant behavioral tests. The methods offer the additional advantage of directly assessing the very element of interest—the level of performance that is observed following medical treatment, threat agent exposure, or their combination. The use of large animal species closely related to humans (such as nonhuman primates) further bolsters the predictability and utility of such behavioral testing methods.

The present report is comprised of three experiments. Experiment 1 characterized the basic progression, time course, and severity of carfentanil intoxication by cage-side observation in male African green monkeys, using an up-down dosing design to determine the ED_{50} for bradypnea/loss of posture. Experiment 2 characterized the basic progression, time course, and degree of recovery provided by naloxone (IM) following a fixed challenge dose of carfentanil, using an up-down dosing method in an attempt to determine an ED_{50} for prompt (< 10 min) and complete reversal of carfentanil intoxication. Additionally, a small dose range-finding study was conducted to explore the rapidity of reversal of carfentanil intoxication following high dose ($\sim$ 1 mg/kg) naloxone (IM). Experiment 3 characterized carfentanil intoxication and the dose-response, degree, and time course of recovery provided by naloxone (IM) at two treatment times, using choice reaction time operant behavior performance. The safety of naloxone alone was also evaluated with these same methods.

2. Methods

2.1. Subjects

Fourteen experimentally experienced adult male African Green monkeys (*Chlorocebus aethiops sabeus*) (5.74–6.64 kg, mean 6.14 kg) of Caribbean origin were individually housed in stainless steel squeeze-back one-over-one nonhuman primate housing units that could be interconnected laterally to additional housing units of identical configuration. Each unit consisted of two vertically stacked cages, with each cage having an automated watering system and an effective area of 82 cm W X 82 cm D X 85 cm H. The colony was maintained at 21 ± 2 °C with a relative humidity of 50 % ± 15 % on a 12 h light/dark cycle (lights on at 0600). Allotted food (Certified Primate Diet 5048, Purina Mills, Inc., St. Louis, MO), provided via a cage top feeding style and supplemented daily with fresh fruit and vegetables, was controlled to maintain healthy body weights. Water was available *ad libitum*. Animals had continual visual and auditory access to conspecifics and were provided with a variety of enrichment devices (e.g., mirrors, chew toys, manipulanda, foraging boards). During experimentation (approximately 3 h daily), animals were restricted to the lower half of the two-cage housing unit. On behavioral training and testing days, the food ration was provided at least one hour after testing concluded. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense (USAMRICD) and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The USAMRICD is a research facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Apparatus

The subjects were tested unrestrained in their home cages. Each monkey had a dedicated aluminum panel that was affixed to the front of the home cage via four aluminum hooks during daily behavioral testing. Each panel consisted of a touchscreen monitor (38 cm capacitive flat panel LCD, Model 1547 L, ELO, Inc., Menlo Park, CA), a notebook computer (Dell Latitude C640 running Windows 7) which controlled experimental events and collected data via a custom-written Visual Basic 2013 (Microsoft Corporation, Redmond, WA) software program, and a pellet dispenser (Model ENV-203-190, Med Associates Inc., St. Albans, VT) that delivered food pellets. A mixture of 190 mg precision food pellets (Formula F0158 grain-based banana or F0035 casein-based banana pellets, and Formula 05798 casein-based fruit crunchies, Bio-Serv, Flemington, NJ) was used. The ratio of the mixture was 3:1 fruit crunchies:banana which was based on extensive experience with this primate species in our laboratory. When delivered as a reinforcer for correct responding, the pellet dropped immediately into a food cup centered below the touch screen. During testing, the animal's home cage door was raised and secured to allow unhindered access to the touch screen and food cup.

2.3. Choice reaction-time (RT) test

An automated operant behavioral test was used to assess neurobehavioral function and the toxicity, safety, and efficacy of carfentanil and naloxone. Each daily session was composed of 240 trials, began with a 3-min delay, and was approximately 120 min in duration. Trials were paced such that each trial was initiated 30 s following the initiation of the previous trial presenting two trials per min irrespective of responding (i.e., the time between trials adjusted dynamically to maintain trial pacing). Each trial is best described as a chained schedule (Ferster and Skinner, 1957), with the presentation of the terminal link contingent upon first satisfying the response requirement of the initial link. The initial link began with the presentation of a 5 cm gray square in a random location upon the touchscreen. Completion of a fixed-ratio 5 response requirement (i.e., touching the square five times) within the 10 s limited hold terminated the stimulus and initiated an unpredictable delay ranging from 0.25 to 1.5 s. Once the random delay elapsed, two new targets appeared in random locations on the touchscreen. Each target was an identical 5 cm gray square except the correct target had a large white circle in the center, whereas the incorrect target (foil) had a large black "X" in the center. A single response to the correct terminal-link target within a 2.4 s limited hold always produced the conditioned reinforcer (a 0.25 s flash of white on the screen and illumination of the food cup), but delivery of the food pellet (primary) reinforcer was scheduled randomly with a probability set between .333–.500, depending on the monkey. This intermittent reinforcement schedule was used to maintain high levels of responding and reduce the likelihood of satiation. A response to the incorrect target or failure to respond within the terminal link's 2.4 s limited hold terminated the trial without any (conditioned or primary) reinforcer. The screen was always blank during the period between trials, between links within a trial, and before and after the session.

2.4. Chemicals

Carfentanil citrate (2-hydroxypropane-1,2,3-tricarboxylic acid; methyl 1-(2-phenylethyl)-4-(N-propanoylanilino)piperidine-4-carboxylate; approximately 98 % purity) obtained from the U.S. Army Edgewood Chemical Biological Center was dissolved in sterile water. Stock

solutions of carfentanil were kept at 4 °C. On the day of experimentation, the stock solution of carfentanil was aliquoted, and additional sterile water was added to achieve a final concentration of 22.5 μg/mL to keep injection volumes at or below 0.35 mL. Naloxone HCl ((4R,4aS,7aR,12bS)-4a,9-dihydroxy-3-prop-2-enyl-2,4,5,6,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7-one hydrochloride) injection solution (0.4 mg/mL), USP was obtained from Hospira, Inc. (Lake Forest, IL, USA). Naloxone HCl dihydrate ((4R,4aS,7aR,12bS)-4a,9-dihydroxy-3-prop-2-enyl-2,4,5,6,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7-one hydrochloride dihydrate) was obtained from Sigma-Aldrich (St. Louis, MO; ≥98 % purity; N7758). The naloxone salt was added to sterile physiological saline (0.9 %) and passed through a 0.1 μm filter into a sterile vial. Stock solutions of naloxone (up to 40 mg/mL) were made on a weekly basis and kept at 4 °C. On the day of experimentation, an aliquot of naloxone was obtained from the stock solution and diluted to the desired concentration (1 − 40 mg/mL) to keep injection volumes at or below 0.55 mL. The commercially available formulation, naloxone HCl solution, was used in early experiments (Experiment 2, described below), but the concentration was too low to allow for its use when investigating higher doses, so the naloxone HCl dihydrate (with appropriate adjustments based on slight differences in molecular weight) was used more extensively in these studies. All doses are expressed as the weight of the salt.

2.5. Experiment 1: Determination of the carfentanil ED_{50} for severe intoxication

The goal of this experiment was to evaluate the progression of carfentanil intoxication and to determine the subcutaneous median effective dose (ED_{50}) of carfentanil for producing loss of posture or bradypnea (respiration rate ≤ 10 breaths/min) in this nonhuman primate model. The up-and-down dosing method of Dixon and Massey (1983) was used. Based upon the only available *in vivo* reference using nonhuman primates (Port et al., 1984), the initial dose was set to 0.5 μg/kg. The slope of the dose-response curve was estimated to be 10; thus the interval between doses was set to 0.1 $\log_{10}$. Carfentanil was administered by subcutaneous injection on the back (e.g., between the scapulae) or flank (e.g., lateral region of the torso) at a volume < 0.5 mL while the animal was briefly restrained using the cage squeeze-back mechanism. Following injection, the animal was promptly released and observed for toxic signs continuously during the first hour post-exposure and at hourly intervals thereafter for up to 6 h. Observers were aware of the carfentanil dose. In instances of respiratory distress, the animal was administered naloxone (IM) until overt signs of opioid toxicity (including compromised respiration) were no longer present. Respiration was assessed by visual observation of chest and/or abdominal movements. Respiration rate was determined by counting the number of chest and/or abdominal movements that occurred during an observation window, typically 1 − 2 min. In instances where respiration rate was difficult to ascertain (e.g., shallow breathing, obscured view, etc.), multiple observations were often taken by the same observer and, more typically, multiple observers took observations either simultaneously or sequentially to verify respiration rate.

2.6. Experiment 2: Estimation of the naloxone ED_{50} for reversing carfentanil intoxication

Following the determination of the ED_{50} of carfentanil for producing loss of posture/bradypnea (calculated to be 0.708 μg/kg, SC), a second up-and-down dosing experiment was conducted in an attempt to estimate the ED_{50} of IM naloxone for reversing intoxication produced by a 1.6 x ED_{50} of carfentanil (1.15 μg/kg, SC). All doses of naloxone were administered IM into the lateral thigh. The range of doses was intended to overlap and exceed estimated human equivalent doses (HED) commonly used for the emergency treatment of opioid overdose (e.g., Evzio 0.4 mg, 2.0 mg). The initial dose of naloxone was 38.87 μg/kg, and the interval between doses was set to 0.1 $\log_{10}$. Naloxone was administered following the onset of bradypnea (≤ 10 breaths/min) or prostration, whichever occurred first. The criterion for the successful reversal of carfentanil intoxication was the consumption of two primate biscuits within 10 min following the administration of naloxone.

Table 1
Naloxone doses administered to the nonhuman primates and the corresponding human equivalent doses based on the FDA's Body Surface Area scaling.

Nonhuman Primate Dose (μg/kg)	Human Equivalent Dose (mg)
71.02	2
142.04	4
355.12	10
710.24	20
1420.48	40
2840.96	80

2.7. Experiment 3: Behavioral toxicity of carfentanil and the behavioral safety and efficacy of naloxone

This experiment utilized a sensitive and quantitative behavioral assessment to characterize the time course of carfentanil intoxication and the efficacy of naloxone in attenuating carfentanil intoxication and restoring behavioral function. The behavioral safety of naloxone alone was evaluated using identical methods. Six different doses of naloxone were examined (71.02–2840.96 μg/kg), as depicted in Table 1. These doses were calculated, according to the FDA's body surface area scaling formulas, to be equivalent to 2 − 80 mg doses in a 70 kg human. These HED values are also shown in Table 1. Six extensively trained monkeys were used for these behavioral assessments, and the monkeys not only were trained to stable and consistent levels of operant performance but were also well habituated to the brief manipulations and injections necessary to accomplish the experimental assessments. Monkeys were allocated to naloxone doses according to an incomplete block design, such that at least four animals received each dose of naloxone. Non-injection control days were generally conducted on Mondays and Wednesdays, and vehicle injection control days were conducted on Thursdays. Naloxone-only injection days were generally conducted on Fridays and carfentanil injection days (with or without naloxone) were generally conducted on Tuesdays. When carfentanil challenge was followed by naloxone administration, the carfentanil challenge dose equaled 1.15 μg/kg SC. When naloxone was not administered, a *lower* carfentanil challenge dose of 0.575 μg/kg SC was used to produce intoxication while also reducing the possibility of death on these carfentanil-only (no treatment) days. The timing of post-carfentanil naloxone administration was evaluated at two separate times: 8 min post-carfentanil ("Immediate Treatment" intended to represent early treatment—such as self-aid and/or upon self-report of symptoms—given at the average time to the onset of mild signs like yawning, resting forehead on the cage, staring, and/or slow blinking) and at the onset of bradypnea or loss of posture for each individual monkey ("Delayed Treatment" intended to represent buddy aid or emergency response treatment at the onset of these severe and obvious opioid effects). The behavioral safety of naloxone was evaluated by administering sterile water (SC; carfentanil vehicle) followed 8 min later by naloxone. The monkey was briefly restrained using the cage squeeze-back mechanism for the carfentanil (or vehicle) SC injection. Following the carfentanil (or vehicle) injection, toxic signs were recorded for the duration of the inter-injection interval. At the appropriate time or toxic sign, the animal was again briefly restrained using the squeeze-back mechanism for the IM naloxone (or vehicle) injection. The pre-session delay (equal to 3 min) was initiated on the laptop computer that controlled the behavioral session, the behavioral panel was hung on the home cage, and research personnel promptly exited the colony room. Each monkey's

activity and posture were monitored via closed circuit video while operant behavior was evaluated continuously for two hours on the automated Choice RT task described above.

2.8. Statistical analyses

The median effective doses (ED_{50}) were calculated according to the method of Dixon and Massey (1983). Although several measures of performance were recorded, only one measure (choice accuracy) is presented herein because it is the most sensitive indicator of integrated behavioral function, capturing performance in each link of the choice RT test, and economically summarizing overall operant proficiency (cf. Moffett et al., 2018). Linear mixed-effects (LME) models were fitted to choice accuracy using the lmer function of R (R for Windows version 3.2.5, R Foundation for Statistical Computing, Vienna, Austria; 'lme4' package version 1.1–12). The analyses of choice accuracy examined the time course of naloxone's effects and were conducted by first aggregating the 240 trials of each session into 24 blocks of 10 trials each, with each block corresponding to a 5-min period. Three separate linear mixed-effects models were fitted, one for each dose-response curve generated: naloxone alone, the efficacy of immediate naloxone administration, and the efficacy of delayed naloxone administration. For all LME analyses, naloxone dose and block were treated as fixed effects and subject was treated as a random effect. Significant main effects were followed by pairwise comparisons between all levels of the fixed-effects. Significant interactions were followed by step-down analyses examining simple main effects. Pairwise comparisons were conducted with the lsmeans function ('lsmeans' package, version 2.25–5), and adjustments for multiple comparisons were made using Bonferroni's correction procedure. A significance level of $p < .05$ was used for all tests.

3. Results

3.1. Experiment 1: determination of the carfentanil ED_{50} for severe intoxication

A total of eleven animals were evaluated across a range of doses of carfentanil (0.5–1.0 μg/kg) before the stopping criterion of four reversals was achieved. The estimated ED_{50} for intoxication, calculated according to the method of Dixon and Massey (1983), was 0.708 μg/kg (95 % CI: 0.579-0.866 μg/kg). The ED_{50}, calculated from pool adjacent violators algorithm (PAVA) isotonic regression in conjunction with bootstrapped confidence intervals, was 0.706 μg/kg (95 % CI: 0.570-0.842 μg/kg). Fig. 1 shows the fitted probit regression model along with the doses evaluated. The probit model returned a slope of 19.887, and the ED_{50} determined from this model was 0.707 μg/kg. Five animals required treatment with naloxone and one animal died within 70 min of administration of 0.794 μg/kg carfentanil due to cardiorespiratory failure (despite attempts at revival with naloxone therapy and cardiopulmonary resuscitation).

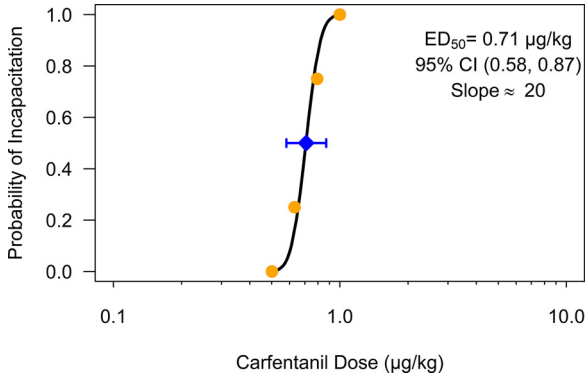

Fig. 1. Dose-effect function for the determination of the carfentanil ED_{50} for incapacitation (the occurrence of bradypnea or loss of posture). Administered doses are indicated by the filled gold circles, the ED_{50} estimate and the 95 % confidence intervals are indicated by the blue filled diamond and error bars, and the probit function is indicated by the solid black line.

3.2. Experiment 2: estimation of the naloxone ED_{50} for reversing carfentanil intoxication

A total of eight animals were evaluated using a fixed challenge dose of carfentanil (1.15 μg/kg), and naloxone was administered upon the occurrence of bradypnea or loss of posture (whichever occurred first). The doses of naloxone ranged from 38.78 to 245.23 μg/kg IM. At the lower doses, beneficial effects upon respiration occurred, but opioid intoxication was often observed to reappear within a few hours or less, often necessitating the administration of additional naloxone. Similarly, at low initial doses of naloxone, the apparent completeness of recovery was judged to be inadequate (mild sedation was still present, monkeys behaved abnormally, etc.), and additional naloxone was administered before the end of the work day/observation period. However, overall, no clear relation was observed between the dose of naloxone and the rapidity of reversal of carfentanil intoxication based on our *a priori* defined treatment success criterion (i.e., consumption of 2 primate biscuits within 10 min of naloxone administration). Similarly, based on basic functioning (e.g., increased respiration, regaining posture, moving up to the perch, etc.) naloxone dose did not predict the time required for the resumption of normal function. Fig. 2 shows the time-course of carfentanil (1.15 μg/kg) intoxication. The time of occurrence (Y axis) of a particular clinical sign (X axis) is indicated for each individual monkey by a unique color-symbol combination in Fig. 2 (an identical color-symbol is used in Fig. 3). As seen in Fig. 2, the general progression of signs was typical across animals, and early signs tended to occur quickly for nearly all animals, but substantial inter-subject variability in the latency to carfentanil-induced respiratory depression and bradypnea was observed. Loss of posture and bradypnea tended to co-occur when both were observed in monkeys.

Fig. 3 shows the time-course of the reversal of carfentanil intoxication following naloxone administration. (Recall that the naloxone dose differed across animals, and this figure is intended to show general progression of naloxone's reversal of opioid intoxication, not dose-dependent effects.) The average time to naloxone treatment following carfentanil was 27.64 min (range: 7 − 56 min). For the majority of animals, reversal of carfentanil intoxication, as indexed by observational methods, was complete by approximately 40 min post-treatment with a few exceptions (normal respiration and normal appetence). Due to the lack of clear dose relationship on promptness of recovery, three additional animals were administered higher doses of naloxone (1.07–1.3

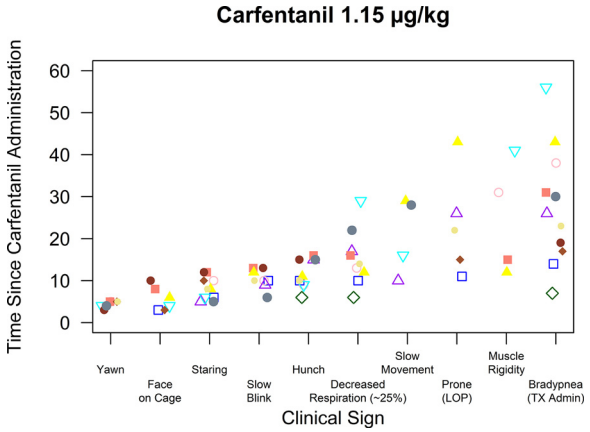

Fig. 2. Latency (in minutes) to onset of various signs of carfentanil intoxication. Individual monkeys are represented by a unique symbol-color combination.

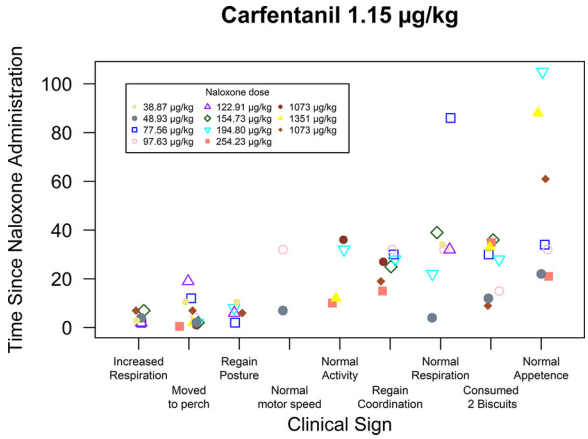

Fig. 3. Latencies (in minutes) to the reversal of carfentanil intoxication. Naloxone doses ranged from 39 µg/kg to 1351 µg/kg. Individual monkeys are represented by a unique symbol-color combination consistent with those in Fig. 2.

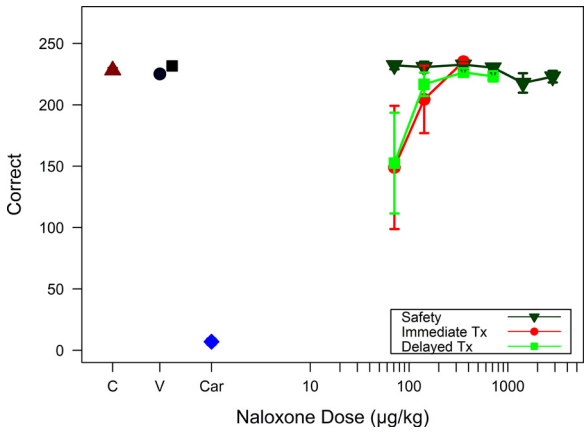

Fig. 4. Correct choice reaction-time responses as a function of naloxone dose or experimental condition. C = non-injection control; V = Vehicle-injection control (filled black circle – immediate treatment; filled black square – delayed treatment). Error bars represent ± SEM and where not visible, error bars are subsumed by symbol. Each symbol represents the average of 4-6 animals.

mg/kg IM) to evaluate whether higher doses would result in more rapid reversal of intoxication. Recovery did occur in all three monkeys; however, both subjects receiving the 1.07 mg/kg dose regurgitated within 5 min of naloxone administration. Moreover, there was still no clear relationship between the dose of naloxone and the rapidity of consuming two primate biscuits nor in the return of other normal behavioral functions, suggesting that above a certain point, increased initial doses of naloxone may confer little or no additional benefit.

3.3. Experiment 3: behavioral toxicity of carfentanil and the behavioral safety and efficacy of naloxone

To overcome some of the prior experiments' shortcomings (e.g., the subjective nature of the evaluations, the lack of replication in naloxone dose and timing of treatment), Experiment 3 was undertaken to utilize sensitive and objective performance measures with consistent and repeatable assessment methods while utilizing the important information gained in Experiments 1 and 2. Monkeys were extensively trained to high levels of performance on the Choice RT task described above and habituated to a variety of control procedures (brief restraint and SC and IM injections with varying inter-injection intervals) prior to formal data collection. Important within this experiment was the inclusion of appropriate control conditions with vehicle injections and the corresponding non-injection condition. These conditions formed the basis of comparison for evaluating effects of the injection procedures *per se* (non-injection vs. vehicle injection), the safety of naloxone (safety data at each naloxone dose vs. vehicle injection), carfentanil toxicity (carfentanil vs. vehicle injection), and the efficacy of each naloxone dose based on early or later treatment (Immediate treatment and Delayed treatment compared to vehicle injection and Carfentanil). Fig. 4 shows the group average (± SEM) number of correct choice responses made during each 2 -h session as a function of the various experimental conditions and dose of naloxone. As seen in this figure, the vehicle injection itself produced no discernible effect on performance compared to non-injection sessions. Carfentanil (0.575 µg/kg) alone produced a nearly complete suppression of behavior. (In many cases the error bars are completely subsumed by the symbols for a given condition, indicating the low levels of variability observed.)

In the immediate treatment condition, an approximate 40 % decrease in the number of correct responses was observed for the session following treatment with the lowest dose of naloxone (71 µg/kg). Similarly, an approximate 15 % suppression of performance relative to baseline levels was observed for the session following immediate treatment with naloxone at 142 µg/kg. Near-baseline levels of performance were observed in all other conditions in which naloxone was administered (either alone or following carfentanil challenge).

Statistical analyses were conducted to verify the visual similarities and differences observed. A linear mixed-effects model was fitted to the sum of correct choice responses by block and treatment condition (i.e., non-injection control, vehicle-injection control, and three doses of naloxone; 71, 142, and 355 µg/kg) in the immediate treatment condition resulted in a significant main effect of naloxone dose, $F(4,452.79) = 62.26$, $p < .0001$. The main effect of block [$F(23,451.01) = 0.82$, $p > .69$] and the interaction between block and treatment condition [$F(92,451.01) = 0.33$, $p = 1.0$] were not significant. Pairwise comparisons between treatment conditions revealed no significant differences in correct choice responses between non-injection and vehicle injection ($p \sim 0.18$) conditions. Correct choice responses following immediate treatment with 71 µg/kg of naloxone were significantly lower than those under non-injection, vehicle injection, 142, and 355 µg/kg naloxone. Additionally, correct choices following immediate treatment with 142 µg/kg were significantly lower than those under non-injection as well as those following immediate treatment with 355 µg/kg. Thus, a dose-dependent benefit of naloxone treatment was evident in the immediate treatment condition (wherein naloxone was administered 8 min after carfentanil challenge), with the highest dose studied (355 µg/kg) providing the best functional behavioral outcome.

Fig. 5 shows the dose-response effects of naloxone on the group average (± SEM) of the summed correct choice responses as a function of trial block. As shown in this figure, correct choice responses following the administration of carfentanil (0.575 µg/kg) in the absence of naloxone began at about 50 % in the first block, declined precipitously, and were almost completely abolished by the third block of 10 trials, approximately 25 min after carfentanil administration. Immediate treatment with 71 µg/kg naloxone produced incomplete recovery of correct choice responding, with a visual trend of slightly decreasing correct choice responses across the entire session. Immediate treatment with 142 µg/kg naloxone produced complete or near-complete recovery for approximately the first half of the session, but correct choice responding during the last half of the session approximated only 80 % of the vehicle injection condition. Performance following immediate treatment with 355 µg/kg was equivalent to that following vehicle injection and remained high for the entirety of the session. Thus, immediate treatment with naloxone afforded prompt reversal of carfentanil intoxication, but the degree of the reversal and the sustainment of the beneficial effect was dose dependent. Only the 355 µg/kg dose provided rapid, complete, and sustained reversal of carfentanil intoxication.

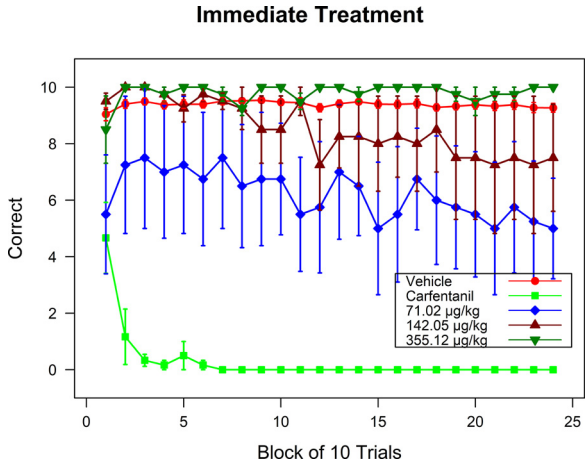

Fig. 5. Correct reaction-time responses collapsed across blocks of 10 trials and animals (n = 4-6/point) when naloxone (71, 142, or 355 µg/kg) was administered at a fixed time (8 min) following carfentanil administration. Error bars are ± SEM and where not visible, error bars are subsumed by the symbol.

3.4. Behavioral efficacy of delayed treatment with naloxone

In the delayed treatment condition, four doses of naloxone (71 − 710 µg/kg) were administered following the onset of bradypnea or loss of posture, whichever occurred first. The average time to naloxone administration was 23.5 min (10.25 min [SD]; 10–40 range) after carfentanil administration. The dose-response effects of naloxone on correct choice responses in the delayed treatment condition are presented in Fig. 4 and denoted by the filled green squares and connecting line. A linear mixed-effects model fitted to the sum of correct choice responses by block of trials revealed a significant main effect of condition, $F(5, 525.1) = 59.91$, $p < .0001$. Pairwise comparisons revealed that delayed treatment with 71 µg/kg naloxone resulted in significantly fewer correct choice responses than in all other treatment conditions (non-injection, vehicle injection, 142 µg/kg, 355 µg/kg, and 710 µg/kg naloxone).

Fig. 6 shows the time course and dose-response effects of naloxone efficacy in reversing the effects of carfentanil intoxication on correct choice responses. As seen in this figure, delayed treatment with 71 µg/kg of naloxone was partially and incompletely effective in reversing the effects of carfentanil intoxication on the behavioral performance of African green monkeys. The linear mixed-effects model fitted to the sum of correct choice responses during each 10-trial block comparing non-injection, vehicle injection, and four doses of naloxone (71 − 710 µg/kg) in the delayed treatment condition revealed a significant main effect of block, $F(23,523.06) = 8.22$, $p < .001$, and a significant interaction between condition and block, $F(115,523.06) = 1.72$, $p < .0001$. Pairwise comparisons of block revealed that correct choice responses made during the first block were significantly lower than those made during blocks 2-24. Pairwise comparisons (test of simple main effects) of treatment condition (i.e., non-injection, vehicle injection, doses of naloxone) during each block of 10 trials revealed that correct choice responses during the *first block of trials* following delayed treatment with all doses of naloxone were significantly lower than those during both the non-injection and vehicle injection conditions. During the *second block of trials*, delayed treatment with 355 or 710 µg/kg resulted in performance that was statistically indistinguishable from non-injection and vehicle injection conditions; however, treatment with 71 and 142 µg/kg resulted in significantly fewer correct choices compared to both non-injection and vehicle injection conditions. This result affirms that the rapidity of functional recovery following delayed treatment can be enhanced with higher doses of naloxone. Correct choice responses following delayed treatment with 71 µg/kg naloxone were significantly lower than those made following non-injection and vehicle injection conditions during blocks 1–2 and 15–24, following treatment with 142 µg/kg naloxone during blocks 16–18 and 20–24; following treatment with 355 µg/kg naloxone during blocks 16–24; and following treatment with 710 µg/kg naloxone during blocks 2 and 15-24. In short, the 71 µg/kg dose was clearly inferior to other (higher) doses in providing prompt and sustained reversal of the carfentanil-induced performance impairments.

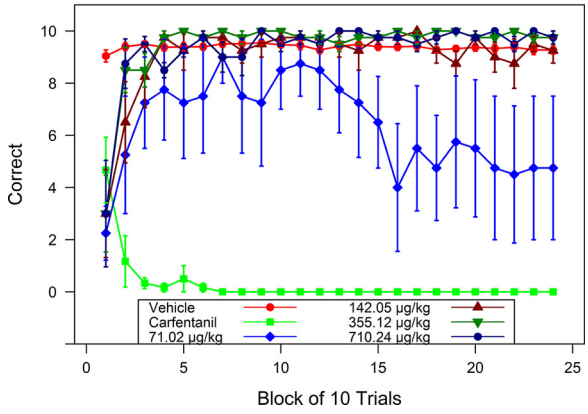

Fig. 6. Group mean correct responses (± SEM) across blocks of 10 trials as a function of experimental condition (n = 4-6/point). Doses of naloxone spanned a 10-fold range (71-710 µg/kg) and were administered at the onset of bradypnea or loss of posture. For other details see caption of Fig. 5.

3.5. Behavioral safety of naloxone

The dark green inverted triangles in Fig. 4 show the dose-response effects of naloxone (71 − 2841 µg/kg) alone on the number of correct choice RT responses (± SEM). Recall that the carfentanil vehicle (sterile water) was given in place of carfentanil for these conditions.

To assess safety, Fig. 7 shows the dose-response effects of naloxone alone on the number of correct choice RT responses by block. As seen in this figure, correct choice RT responding following all doses of naloxone approximated control values with few notable exceptions. There were small decrements in correct choice RT responses at the two highest doses of naloxone; however, under all other conditions, responding approximated the maximum possible. The LME fitted to these data revealed a significant main effect of treatment condition, $F(7,765) = 8.77$, $p < .0001$; however, the main effect of block $F(23,763) = 0.90$,

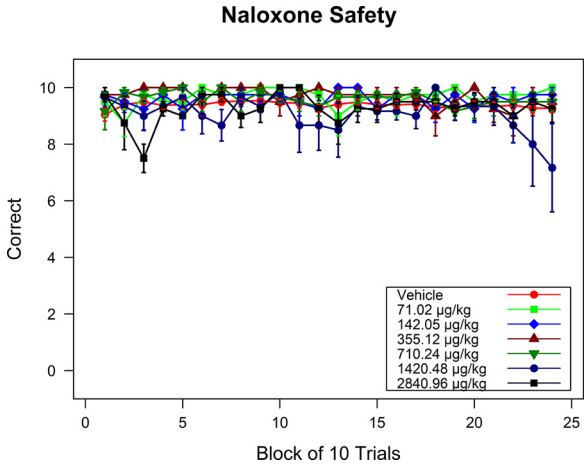

Fig. 7. Correct choice-reaction time responses as a function of blocks of 10 trials and doses of naloxone administered 8 min following vehicle injections (n = 4-6/point). Error bars are ± SEM.

$p > 0.58$ and the interaction between treatment condition and block were not significant, $F(161,763) = 0.82$, $p > .92$. Pairwise comparisons between treatment conditions revealed that correct choice RT responses after 71 μg/kg naloxone were significantly higher than after vehicle injection. Correct choice RT responses following administration of 1420 μg/kg naloxone were significantly decreased compared to those made following non-injection (data not shown), vehicle injection, and 71 − 710 μg/kg naloxone. Likewise, correct choice RT responses following administration of 2841 μg/kg naloxone were significantly decreased compared to 71 μg/kg, 355 μg/kg, and 710 μg/kg naloxone. The latter two findings appear to support the idea that these higher naloxone doses may produce subtle behavioral impairments when given alone.

4. Discussion

The present experiments represent the first of their kind: well-controlled laboratory studies to characterize the time course and progression of toxic signs following carfentanil injection in nonhuman primates. These experiments also evaluated a range of naloxone doses to reverse carfentanil intoxication. Further refinements were made to those assessments through the use of operant behavioral procedures to directly measure the functional impairment caused by carfentanil, the efficacy of naloxone to reverse this impairment (when administered at early and late signs of intoxication), and the safety of naloxone when given alone. The inclusion of early range-finding and observational studies followed by more definitive and quantitative studies resulted in data sets that can be used to make assessments and comparisons of treatment outcomes across this broad range of naloxone doses, while also appreciating drug safety in this same nonhuman primate behavioral model.

Experiment 1 determined the SC ED_{50} of carfentanil for bradypnea and/or loss of posture in the male African green monkey to be 0.71 μg/kg (95 % CI: 0.58-0.87 μg/kg). This estimate aligns quite well with the limited experimental data that exist for carfentanil in other laboratory studies in nonhuman primates (Port et al., 1984). In their study using female rhesus monkeys, respiratory depression was produced at doses of 0.6 and 1.0 μg/kg IV. Although the progression of signs was not as carefully documented as in the present study, the initial intoxicating effects were generally observed within 5 − 10 min, as in the present study, despite the difference in route of exposure. Importantly, based upon the somewhat protracted development of intoxication from mild to severe effects observed in that study and in the present study, the opportunity may exist for self-report and/or self-aid to occur following carfentanil exposure and early intoxication in humans. Indeed, the progression from mild to severe intoxication took approximately 15 min in the present study. The extent to which this time course of intoxication may be altered by route of opioid exposure and/or the dose administered remains a topic for further evaluation.

Although Experiment 2 failed to meet its stated objective of establishing an ED_{50} of naloxone for rapidly reversing functional impairments produced by a fixed dose of carfentanil (1.15 μg/kg SC or 1.6 X ED_{50} for bradypnea/loss of posture), this experiment actually met the more qualitative goals for which it was intended, namely, to determine a range of naloxone doses that could be evaluated more definitively in Experiment 3. Experiment 2 selected, *a priori*, the success criterion that each monkey consume two primate biscuits within 10 min of naloxone administration. However, this criterion was not routinely met even under the most (otherwise) successful treatment doses, rendering the criterion too stringent to be useful. Despite this overly strict criterion, other observational outcomes following varying doses of naloxone were indispensable in providing information necessary for Experiment 3 and that could not have been garnered otherwise. Specifically, the failure of low doses of naloxone (i.e., less than 71 μg/kg or the 2 mg HED) to provide a complete or sustained recovery precluded their inclusion in Experiment 3. On the other extreme, high doses of naloxone (710 μg/kg or 20 mg HED and higher) appeared to confer little additional benefit, and this information further honed the range of treatment doses evaluated in Experiment 3. Additionally, the time-dependent treatment parameters for early/mild ("Immediate treatment") and late/severe ("Delayed treatment") medical intervention with naloxone used in Experiment 3 were determined based upon the copious observations within Experiment 2.

Experiment 3 utilized operant behavioral methods to evaluate the effects of carfentanil (0.575 μg/kg) alone, the safety of naloxone (71–2841 μg/kg), and the efficacy of naloxone (71 − 710 μg/kg) therapy when administered at two different times (8 min after 1.15 μg/kg carfentanil injection or at the onset of carfentanil-induced bradypnea/loss of posture) on a well-trained food-reinforced choice reaction time procedure. Experiment 3 determined that carfentanil alone, even at the lower dose of only 0.575 μg/kg, produced prompt and nearly complete suppression of the operant response measures. With the higher carfentanil challenge dose (1.15 μg/kg) and under both the immediate and delayed treatment times, naloxone administered at doses of 71 and 142 μg/kg (2 and 4 mg HED) only partially or briefly reversed the carfentanil-induced behavioral suppression. In contrast, naloxone doses at or above 355 μg/kg (10 mg HED) provided complete reversal of carfentanil effects on choice reaction-time performance sustained throughout the 2 h behavioral assessment period following both treatment times. In the delayed treatment condition, the latency to fully recover behavioral performance was dose-dependent but required approximately 15 − 20 min at the most efficacious doses. Based on the time course data, the functionally effective naloxone dose for the treatment of carfentanil intoxication across both treatment conditions (immediate and delayed) was 355 μg/kg. Behavioral safety assessments in these same animals indicated that naloxone is clearly safe at doses up to 710 μg/kg, with safety concerns increasing as doses approach 1420 and 2841 μg/kg (40 and 80 mg HED, respectively), having the possibility of degrading behavioral performance even in the absence of opioid poisoning. These results have practical relevance for the selection of opioid antagonists, initial doses, and expected functional outcomes following treatment of synthetic opioid overdose in a variety of operational/emergency-response contexts. The safety data further suggest that there may be practical safety limits to potential prophylactic administration of naloxone as well.

The protracted (2 h) behavioral sessions provided detailed time course data on the efficacy of naloxone and indicated that the rate of naloxone elimination may be faster than that of carfentanil (Figs. 5 and 6). There have been few evaluations of the pharmacokinetics of carfentanil in non-humans and only one in humans (Minkowski et al., 2012). The reported half-life of carfentanil in humans was approximately 45 min (Minkowski et al., 2012). In contrast, the reported half-life of IM naloxone (EVZIO, 2 mg) in humans was 90 min (cf. EVZIO package insert; Full Prescribing Information; Reference ID: 4001455). In the absence of pharmacokinetic data in African green monkeys, the results of the current studies indicate that differences in pharmacodynamic effects and/or receptor binding affinities may be responsible for the partial protection/recovery afforded by naloxone at doses of 71 and 142 μg/kg.

Although the present study did not generate a dose-response curve for carfentanil on choice reaction-time performance, previous studies with non-human primates have examined the effects of opioids and naloxone on food reinforced operant behavior. Several related opioids have been examined more extensively using schedule-controlled responding for food. The effects of heroin (10–1000 μg/kg), morphine (32–10000 μg/kg), and fentanyl (1–100 μg/kg) on schedule-controlled responding for food have been examined in rhesus monkeys (Downs and Woods, 1976; Lukas et al., 1986; Negus et al., 2009, 2003; Negus et al., 1993; Stevenson et al., 2003, 2005). Reported ED_{50} values for the suppression of schedule-controlled behavior of rhesus monkeys for heroin ranged from 88 to 170 μg/kg (Negus et al., 2003; Stevenson et al., 2005). Similarly, reported ED_{50} values for morphine's

suppression of schedule-controlled behavior in rhesus monkeys ranged from approximately 1000 μg/kg (Downs and Woods, 1976) to 2300 μg/kg (Stevenson et al., 2003). Finally, ED_{50} values for fentanyl's suppression of the schedule-controlled behavior of rhesus monkeys ranged from 6.6–23 μg/kg ((Negus et al., 2009, 1993; Stevenson et al., 2003).

Downs and Woods (1976) examined the effects of morphine and naloxone on the performance of rhesus monkeys responding under a multiple fixed-ratio 30 fixed-interval 5 min schedule of food reinforcement. Morphine was evaluated over the range of 32 − 10000 μg/kg whereas naloxone was assessed across the range of 0.032 − 32 mg/kg. Morphine, at doses above 5600 μg/kg, completely suppressed response rates on both the fixed-ratio and fixed-interval components. Although not reported in the original manuscript, the approximate ED_{50} value of morphine for the suppression of response rates in the fixed-ratio component was 1000 μg/kg, whereas for the fixed-interval component the approximate ED_{50} of morphine was 560 μg/kg. Moreover, the approximate ED_{50} of naloxone for response rate suppression under both the fixed-interval and fixed-ratio components was 32.0 mg/kg. Subsequently, that study evaluated the effects of morphine (1000 − 10000 μg/kg)-naloxone (0.003–3.0 mg/kg) interactions on multiple-schedule performance. In general, increasing doses of morphine required increasing doses of naloxone to restore multiple-schedule performance to control levels. However, a complex interaction between the component schedule of reinforcement and the doses of naloxone and morphine was apparent in that the dose of naloxone appeared to be more effective in antagonizing morphine's effects on fixed-ratio performance. For example, when the 10,000 μg/kg morphine was administered, 0.32 mg/kg naloxone restored performance to near control levels under the fixed-ratio component; however, 3.2 mg/kg naloxone was required to restore performance to control levels under the fixed-interval component, suggesting a possible rate dependency (McKearney, 1981).

Goldberg et al. (1981) examined the effects of acute and chronic treatment with naltrexone and naloxone on the multiple schedule controlled behavior of squirrel monkeys and pigeons. In squirrel monkeys, naloxone at doses ≥ 3 mg/kg caused decreased response rates under the multiple fixed-ratio 30 fixed-interval 10 min schedule. When morphine (3000 μg/kg) was administered concurrently with naloxone (0.01 − 10 mg/kg), rates of responding under both components of the multiple schedule approximated control levels only with naloxone doses of 0.32 and 1.0 mg/kg. Similar to the Downs and Woods (1976) study detailed above, when a range of morphine doses (300 − 30000 μg/kg) was administered in combination with a range of naloxone doses (0.01–1.0 mg/kg), higher doses of naloxone were required as the dose of morphine increased to restore responding to control levels. This resulted in rightward shifted dose − response curves for morphine in combination with increasing doses of naloxone. Thus, in general, across a range of opioids, greater doses of naloxone are required to treat higher levels of opioid intoxication, either because the dose is higher or the opioid more potent.

One limitation of the present studies was examining naloxone efficacy against only one challenge dose of carfentanil in the operant behavioral assessment procedure. The 355 μg/kg dose of naloxone proved to be efficacious against the 1.15 μg/kg carfentanil challenge; however, the effect level of this dose of carfentanil is currently unknown. Given the high potency of carfentanil and other fentanyl analogues, the sufficiency of this naloxone dose for immediate or delayed rescue in cases of supra-lethal doses of carfentanil or other fentanyl analogues is unknown and should be evaluated in future studies. Another limitation of the current studies was the exclusive use of male subjects. Females have been shown to respond differently to opioids than males under some conditions (Kest et al., 2000; Sarton et al., 2000). Future studies should evaluate the behavioral effects of carfentanil in females to determine whether sex differences in carfentanil toxicity and/or naloxone efficacy exist. Additionally, although it was possible to make some inferences regarding the kinetics of carfentanil and naloxone based upon the clear time-dependent behavioral effects, more definitive pharmacokinetic characterization may prove helpful in understanding these results. Such kinetic studies would allow us to measure the plasma levels of opioids that produce intoxication and make projections regarding the plasma levels of naloxone necessary to achieve a desired therapeutic effect.

Disclaimer

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Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Barrett, J.E., 2002. The emergence of behavioral pharmacology. Mol. Interv. 2 (8), 470–475. https://doi.org/10.1124/mi.2.8.470.

Dews, P.B., 1956. Modification by drugs of performance on simple schedules of positive reinforcement. Ann. N. Y. Acad. Sci. 65 (4), 268–281.

Dixon, W.J., Massey, F.J., 1983. Introduction to Statistical Analysis. Vol.. 4th edition. McGraw-Hill, New York.

Downs, D.A., Woods, J.H., 1976. Morphine, pentazocine and naloxone effects on responding under a multiple schedule of reinforcement in rhesus monkeys and pigeons. J. Pharmacol. Exp. Ther. 196 (2), 298–306.

Eriksson, O., Antoni, G., 2015. [11C]carfentanil binds preferentially to mu-opioid receptor subtype 1 compared to subtype 2. Mol. Imaging 14, 476–483.

George, A.V., Lu, J.J., Pisano, M.V., Metz, J., Erickson, T.B., 2010. Carfentanil–an ultra potent opioid. Am. J. Emerg. Med. 28 (4), 530–532. https://doi.org/10.1016/j.ajem.2010.03.003.

Goldberg, S.R., Morse, W.H., Goldberg, D.M., 1981. Acute and chronic effects of naltrexone and naloxone on schedule-controlled behavior of squirrel monkeys and pigeons. J. Pharmacol. Exp. Ther. 216 (3), 500–509.

Ingman, K., Hagelberg, N., Aalto, S., Nagren, K., Juhakoski, A., Karhuvaara, S., et al., 2005. Prolonged central mu-opioid receptor occupancy after single and repeated nalmefene dosing. Neuropsychopharmacology 30 (12), 2245–2253. https://doi.org/10.1038/sj.npp.1300790.

Kelleher, R.T., Morse, W.H., 1968. Determinants of the specificity of behavioral effects of drugs. Ergeb. Physiol. 60, 1–56.

Kest, B., Sarton, E., Dahan, A., 2000. Gender differences in opioid-mediated analgesia: animal and human studies. Anesthesiology 93 (2), 539–547. https://doi.org/10.1097/00000542-200008000-00034.

Lian, M., Beckmen, K.B., Bentzen, T.W., Demma, D.J., Arnemo, J.M., 2016. Thiafentanil-azaperone-xylazine and carfentanil-xylazine immobilizations of free-ranging caribou (Rangifer Tarandus Granti) in Alaska, USA. J. Wildl. Dis. 52 (2), 327–334. https://doi.org/10.7589/2015-04-101.

Lukas, S.E., Brady, J.V., Griffiths, R.R., 1986. Comparison of opioid self-injection and disruption of schedule-controlled performance in the baboon. J. Pharmacol. Exp. Ther. 238 (3), 924–931.

Lust, E.B., Barthold, C., Malesker, M.A., Wichman, T.O., 2011. Human health hazards of veterinary medications: information for emergency departments. J. Emerg. Med. 40 (2), 198–207. https://doi.org/10.1016/j.jemermed.2009.09.026.

McKearney, J.W., 1981. Rate dependency: scope and limitations in the explanation and analysis of the behavioral effects of drugs. In: In: Thompson, T., Dews, P.B. (Eds.), Advances in Behavioral Pharmacology Vol. 3 Academic Press, New York.

Minkowski, C.P., Epstein, D., Frost, J.J., Gorelick, D.A., 2012. Differential response to IV

carfentanil in chronic cocaine users and healthy controls. Addict. Biol. 17 (1), 149–155. https://doi.org/10.1111/j.1369-1600.2010.00256.x.

Moffett, M.C., McDonough, J.H., McMonagle, J.D., Myers, T.M., 2018. Behavioral, pharmacokinetic, and cardiovascular evaluation of candidate oximes in African green monkeys. Int. J. Toxicol. https://doi.org/10.1177/1091581818779362. 1091581818779362.

Myers, T.M., Hamilton, L.R., 2011. Delayed match-to-sample performance in African green monkeys (Chlorocebus aethiops sabaeus): effects of benzodiazepine, cholinergic, and anticholinergic drugs. Behav. Pharmacol. 22 (8), 814–823. https://doi.org/10.1097/FBP.0b013e32834d6292.

Negus, S.S., Burke, T.F., Medzihradsky, F., Woods, J.H., 1993. Effects of opioid agonists selective for mu, kappa and delta opioid receptors on schedule-controlled responding in rhesus monkeys: antagonism by quadazocine. J. Pharmacol. Exp. Ther. 267 (2), 896–903.

Negus, S.S., Brandt, M.R., Gatch, M.B., Mello, N.K., 2003. Effects of heroin and its metabolites on schedule-controlled responding and thermal nociception in rhesus monkeys: sensitivity to antagonism by quadazocine, naltrindole and beta-funaltrexamine. Drug Alcohol Depend. 70 (1), 17–27. https://doi.org/10.1016/s0376-8716(02)00331-9.

Negus, S.S., Bear, A.E., Folk, J.E., Rice, K.C., 2009. Role of delta opioid efficacy as a determinant of mu/delta opioid interactions in rhesus monkeys. Eur. J. Pharmacol. 602 (1), 92–100. https://doi.org/10.1016/j.ejphar.2008.11.004.

O'Donnell, J., Gladden, R.M., Mattson, C.L., Kariisa, M., 2018. Notes from the field: overdose Deaths with carfentanil and other fentanyl analogs detected - 10 states, July 2016-June 2017. MMWR Morb. Mortal. Wkly. Rep. 67 (27), 767–768. https://doi.org/10.15585/mmwr.mm6727a4.

Port, J.D., Stanley, T.H., Steffey, E.P., Pace, N.L., Henrickson, R., McJames, S.W., 1984. Intravenous Carfentanil in the dog and rhesus monkey. Anesthesiology 61 (3A), A378.

Rice, D.C., 1988. Quantification of operant behavior. Toxicol. Lett. 43 (1–3), 361–379.

Sarton, E., Olofsen, E., Romberg, R., den Hartigh, J., Kest, B., Nieuwenhuijs, D., et al., 2000. Sex differences in morphine analgesia: an experimental study in healthy volunteers. Anesthesiology 93 (5), 1245–1254. https://doi.org/10.1097/00000542-200011000-00018. discussion 1246A.

Stevenson, G.W., Folk, J.E., Linsenmayer, D.C., Rice, K.C., Negus, S.S., 2003. Opioid interactions in rhesus monkeys: effects of delta + mu and delta + kappa agonists on schedule-controlled responding and thermal nociception. J. Pharmacol. Exp. Ther. 307 (3), 1054–1064. https://doi.org/10.1124/jpet.103.056515.

Stevenson, G.W., Folk, J.E., Rice, K.C., Negus, S.S., 2005. Interactions between delta and mu opioid agonists in assays of schedule-controlled responding, thermal nociception, drug self-administration, and drug versus food choice in rhesus monkeys: studies with SNC80 [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenz yl]-N,N-diethylbenzamide] and heroin. J. Pharmacol. Exp. Ther. 314 (1), 221–231. https://doi.org/10.1124/jpet.104.082685.

Weiss, B., Cory-Slechta, D.A., 2001. Assessment of behavioral toxicology. In: Hayes, A.W. (Ed.), Principles and Methods of Toxicology, 4th ed. Taylor & Francis, Philadelphia, pp. 1451–1528.

Weiss, B., Laties, V.G., 1969. Behavioral pharmacology and toxicology. Annu. Rev. Pharmacol. 9, 297–326. https://doi.org/10.1146/annurev.pa.09.040169.001501.

Wong, B., Perkins, M.W., Tressler, J., Rodriguez, A., Devorak, J., Sciuto, A.M., 2017. Effects of inhaled aerosolized carfentanil on real-time physiological responses in mice: a preliminary evaluation of naloxone. Inhal. Toxicol. 29 (2), 65–74. https://doi.org/10.1080/08958378.2017.1282065.

World Health Organization, 2017. Carfentanil: Critical Review Report. Retrieved from Geneva:. . https://www.who.int/medicines/access/controlled-substances/4.8_Carfentanil.pdf?ua=1.

Yong, Z., Gao, X., Ma, W., Dong, H., Gong, Z., Su, R., 2014. Nalmefene reverses carfentanil-induced loss of righting reflex and respiratory depression in rats. Eur. J. Pharmacol. 738, 153–157. https://doi.org/10.1016/j.ejphar.2014.05.044.