USAMRICD-TR-18-10

Median Lethal Dose Determination of Subcutaneous Carfentanil in Ferrets

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September 2018

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
18 September 2018	Technical Report	01 May 2017 to 30 November 2017

4. TITLE AND SUBTITLE
Median Lethal Dose Determination of Subcutaneous Carfentanil in Ferrets

5a. CONTRACT NUMBER

5b. GRANT NUMBER
CB-3950

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rice, NC, Moffett, MC, Myers, TM,

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
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8350 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-TR-18-10

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Defense Threat Reduction Agency
8725 John J. Kingman Road STOP 6201
Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)
DTRA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Carfentanil is an opioid that is 10,000 times more potent than morphine and 100 times more potent than fentanyl. It produces analgesia, incapacitation, respiratory depression, and death at various doses, but its toxicity has been elaborated only to a limited extent using various laboratory animal models. Because one likely route of carfentanil exposure is inhalation, we sought to elaborate carfentanil toxicity in ferrets to fill a key gap in the research data and set the stage for subsequent studies using other routes of exposure (such as inhalation). The pulmonary structure and function of ferrets resembles that of humans, making the ferret an excellent animal model for investigating inhaled toxicants. The current study exposed water-regulated and unregulated male ferrets to carfentanil via subcutaneous injection, which provides a controlled method of exposure that is also safe and easily achieved. Initial toxic signs were lethargy and ataxia, which progressed to fasciculation and unconsciousness at moderate doses. At higher doses, respiratory distress and death were comorbid with occasional convulsions. The median lethal dose was estimated to be 27.77 μg/kg, and water regulation status did not alter carfentanil toxicity. These data help to elaborate carfentanil toxicity in an animal model that offers advantages for studies of inhaled toxicants, and provide a relevant foundation for ongoing and planned studies of inhalation and intravenous carfentanil exposure and medical treatment of opioid overdose.

15. SUBJECT TERMS

carfentanil toxicity, median lethal dose, routes of exposure, water regulation, ferret

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Todd M. Myers
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	13	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-8380

ACKNOWLEDGEMENTS

The authors wish to thank Benjamin Wong, Bryan McCranor, Justin Tressler, Laura Gooch, and Jennifer Devorak for their support and contributions toward this research effort.

ABSTRACT

Carfentanil is an opioid that is 10,000 times more potent than morphine and 100 times more potent than fentanyl. It produces analgesia, incapacitation, respiratory depression, and death at various doses, but its toxicity has been elaborated only to a limited extent using various laboratory animal models. Because one likely route of carfentanil exposure is inhalation, we sought to elaborate carfentanil toxicity in ferrets to fill a key gap in the research data and set the stage for subsequent studies using other routes of exposure (such as inhalation). The pulmonary structure and function of ferrets resembles that of humans, making the ferret an excellent animal model for investigating inhaled toxicants. The current study exposed water-regulated and unregulated male ferrets to carfentanil via subcutaneous injection, which provides a controlled method of exposure that is also safe and easily achieved. Initial toxic signs were lethargy and ataxia, which progressed to fasciculation and unconsciousness at moderate doses. At higher doses, respiratory distress and death were comorbid with occasional convulsions. The median lethal dose was estimated to be 27.77 μg/kg, and water regulation status did not alter carfentanil toxicity. These data help to elaborate carfentanil toxicity in an animal model that offers advantages for studies of inhaled toxicants, and provide a relevant foundation for ongoing and planned studies of inhalation and intravenous carfentanil exposure and medical treatment of opioid overdose.

INTRODUCTION

Carfentanil is an opioid that is 10,000 times more potent than morphine and 100 times more potent than fentanyl. It produces analgesia, incapacitation, respiratory depression, and death at various doses [1]. Carfentanil has recently garnered the attention of public health officials and first responders due to its availability and misuse as an additive to other drugs [1], such as heroin. The chemical properties and availability of carfentanil also make it a potential terror agent, via ingestion or inhalation. To better understand intoxication following inhalation exposure, an appropriate animal model needs to be used. The pulmonary structure and function of ferrets in many ways resembles that of humans, making the ferret an excellent model for investigating inhalation as a route of exposure [2]. However, the ferret has been somewhat underutilized in scientific investigations. Nevertheless, ferrets have been used to a limited extent in behavioral assessments [3-6] and more extensively and primarily as a model for studies of influenza [7-9]. Laboratory studies of opioids (such as carfentanil) represent a smattering of research with different laboratories using different animal models, routes of exposure, and methods of evaluation [10-13], so additional animal model choices and fundamental information regarding opioid toxicity are beneficial to advancing scientific understanding and practical evaluation of opioid compounds in research settings.

To date, the use of ferrets in opioid research appears to be limited to work accomplished within a single lab. In a series of experiments by Mioduszewki and colleagues, ferrets were given sufentanil either alone or with nalmefene, an opioid antagonist, as an intravenous administration [14-16]. They found that when sufentanil was given alone, akinesia could appear within seconds. Doses required to produce intoxication (median effective dose; ED_{50}) were also small, as loss of righting reflex occurred at 7.8 μg/kg, and both unconsciousness and apnea occurred at 9.1 μg/kg. However, the 24-hour median lethal dose (LD_{50}), calculated via probit regression, was substantially higher, estimated to be 285 μg/kg [14]. When nalmefene was then coadministered with a high dose of sufentanil (LD_{84}), it either blocked or attenuated the opiate effects, depending on the ratio of the two compounds. Higher doses of nalmefene (1:1 ratio) completely negated overt signs of intoxication, whereas the lowest dose of nalmefene (a 17:1 sufentanil:nalmefene ratio) did not prevent the immobilizing effects of sufentanil, but did reduce the incidence of apnea and death [14]. Further examination of a 15:1 sufentanil:nalmefene coadministration regimen revealed that lower doses of sufentanil (≤ 316 μg/kg) had the highest blood-plasma concentrations at 2 minutes post-injection. The higher sufentanil doses (≥ 1000 μg/kg) both peaked at 10 minutes post-injection. Nalmefene coadministration decreased the peak amounts observed in the blood-plasma, but did not change the peak times [15]. When the same authors observed the transmission time of neurons in the brainstem, nalmefene again counteracted the lengthened transmission time observed following sufentanil administration. Interestingly, nalmefene coadministration not only reversed the effects of sufentanil, but also shortened transmission times compared to baseline [16].

Fortunately, the same authors also conducted a more directly relevant study using carfentanil, administered intraperitoneally (IP). Carfentanil produced akinesia at 18.2 µg/kg, loss of righting reflex at 37.9 µg/kg, and apnea at 35.1 µg/kg, and the 24-hour median lethal dose was estimated to be 83.1 µg/kg [17]. Naltrexone, a widely available opioid antagonist, was then coadministered (IP) at various doses. Lethality was no longer observed when 42 µg/kg naloxone was given with 168 µg/kg carfentanil. Higher doses of naloxone increased the latency to and/or decreased the duration of toxic signs. A 1:1 ratio of naloxone to carfentanil also preserved the ferret's righting reflex and prevented the development of apnea [17].

Although carfentanil has been investigated in some species, intoxication and lethality resulting from carfentanil exposure in ferrets are still in need of elaboration. Prior to conducting inhalation exposures of carfentanil, it is best to understand other routes of exposure. Non-inhalation routes are likely to be less variable and provide critical comparisons for inhalation exposures by highlighting differences in latency, severity, and duration of intoxication. Other routes of exposure are also likely to be less costly and easier to conduct, thereby allowing a higher throughput examination of therapeutics. The current experiment exposed ferrets to carfentanil via subcutaneous injection, which not only addresses a gap in the literature, but also provides a suitable comparison for future intravenous and inhalation exposure studies and medical countermeasures assessments. Because we have implemented water regulation to motivate ferrets in an operant behavioral task (in separate studies), we also sought to determine whether water regulation would modulate carfentanil toxicity in this animal model.

METHODS

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 the concentration of interest for each stage (described below).

Subjects

Sixty-four (64) male ferrets were obtained from Marshall BioResources (North Rose, NY). Ferrets were descented, weighed between 1.0-1.2 kg and were 13-15 weeks of age upon arrival. Ferrets were allowed 6 days to acclimate to our facility and housed in groups of 8 in a vivarium under a 12-hour light/dark cycle (lights on 0600). During acclimation, all ferrets had free access to food and water. At the end of acclimation, half of the ferrets (n = 32) were placed into the *water-regulated* group and were given access to water for 5 hours a day (1600 to 2100). All other ferrets (n = 32) were placed

into the *ad libitum* group and continued to have free access to water. Both groups continued to have free access to food through the remainder of the study. Subjects were weighed daily to ensure their weights were at least 90% of a previously published growth curve [18].

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.

Median Lethal Dose Determination

The median lethal dose of subcutaneous carfentanil was determined using a stagewise, adaptive dosing design, where 1-2 ferrets were assigned to 1-3 carfentanil challenge doses per stage. The doses used in the first stage were based upon pilot data from a behavioral assessment, where the doses administered were meant to produce intoxication without death. Doses in subsequent stages were selected based upon the 24-hour survival to doses used in the previous stages. Exposures were conducted until the full range of lethality (0% to 100% survival) was obtained and a probit dose-response model was able to accurately determine the median lethal dose. At each stage, ferrets were randomly assigned to doses, and an equal number of ferrets from the *water-regulated* and *ad libitum* groups were used at each stage to determine if water regulation status differentially affected the median lethal dose. The total number of ferrets exposed to each carfentanil dose is indicated in Table 1. Injection volumes were set at 0.5 mL/kg and given between the shoulder blades of the ferrets, who were briefly restrained in a prone position. At the time of injection, ferrets in the *ad libitum* group weighed between 1160 and 1620 grams (M = 1390.6, SD = 120.6) and ferrets in the *water-regulated* group weighed between 1060 and 1580 grams (M = 1320.0, SD = 131.4).

Table 1. *Number of ferrets exposed to each carfentanil dose as a function of group (water-regulated and ad libitum).*

Dose (µg/kg)	Water Regulated	Ad Libitum	Total
2.37	1	1	2
5.62	2	2	4
13.34	2	2	4
15.49	5	5	10
17.78	5	5	10
23.71	5	5	10
42.17	3	3	6
48.70	5	5	10
56.23	2	2	4
100.00	2	2	4

Statistical Analysis

Median lethal dose estimates and associated 95% confidence intervals were obtained by using a probit model in IBM SPSS Statistics 22.

RESULTS

A probit model was fit to 24-hour survival data across the *water-regulated* and *ad libitum* groups. The models predicted similar median lethal doses (29.19 µg/kg and 26.49 µg/kg, respectively) and were not significantly different. As survival was not affected by water-regulation status, data from both groups were combined, and the probit model fitting was repeated. The resulting combined probit model estimated a median lethal dose (and its 95% confidence interval) of 27.77 (20.89 – 38.16) µg/kg and had a slope of 4.04. The combined probit function and the observed survival proportions are shown in Figure 1.

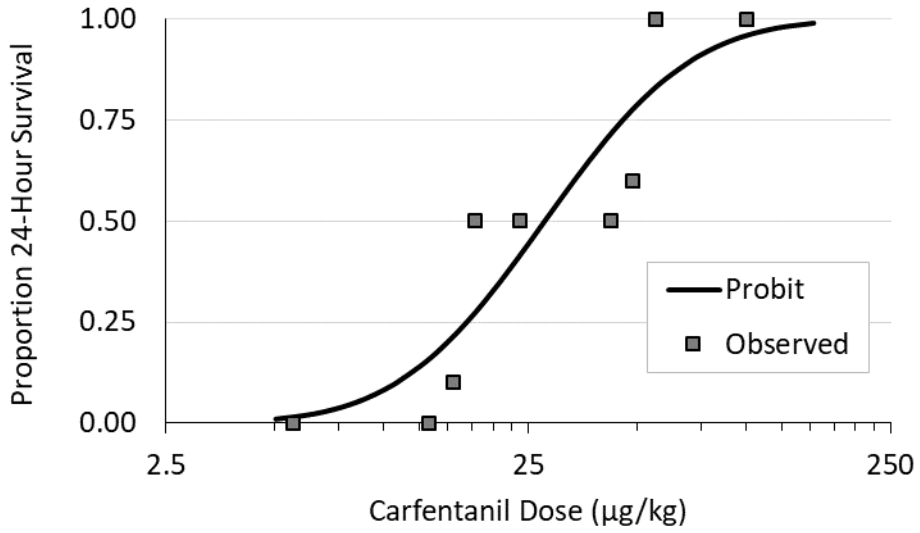

Figure 1. Probit model of 24-hour survival as a function of carfentanil dose (µg/kg). Data shown here combined the *water-regulated* and *ad libitum* groups. Observed survival rates at each dose are shown as gray squares. The estimated median lethal dose was 27.77 µg/kg.

Subjects were observed after exposure, and although the proportion of subjects exhibiting specific toxic signs at specific times was not systematically recorded, the general progression of toxic signs was noted. Lethargy and ataxia were the most common initial signs, though higher carfentanil doses often did not produce overt ataxia due to the rapid and profound onset of lethargy/prostration. Fasciculation and tremor often followed lethargy. Fasciculation was most commonly observed at the site of injection and the face. Unconsciousness often followed, after which the ferrets were observed to have erratic breathing. Breathing often decreased in rate, depth (i.e., shallow breaths), or both. Animals that eventually died typically began gasping at some point and would occasionally have convulsions. The convulsions observed were more akin to shivering and appeared markedly different from convulsions produced by convulsive chemical agents such as acetylcholinesterase inhibitors.

DISCUSSION

The median lethal dose estimated in the current study (27.77 µg/kg) demonstrates the potency of carfentanil and its potential use as a terror agent. The fact that the median lethal dose estimates for the *water-regulated* and *ad libitum* groups were similar is also noteworthy. Ferrets in the *water-regulated* group had no access to water for 15 hours prior to carfentanil exposure. While the hydration status of the ferrets in the *ad libitum* group cannot be determined, as water was available at all times and the time since their last drink is unknown, it is reasonable to assume that they were more hydrated than their water-regulated counterparts. However, regardless of the hydration status of the ferrets, the median lethal dose estimates were equivalent, and no differences in prevalence of toxic signs were observed between the groups.

The median lethal dose from the current experiment is also 3 times lower than that of the IP estimate, 83.1 µg/kg [17]. While different routes of exposure are expected to produce differences in toxicity, definitive comparisons are difficult to make with only two studies. The toxic signs observed here also align with those seen by other researchers. Onset of the milder signs (i.e., ataxia and lethargy) was often rapid, with progression to more severe intoxication at higher doses. One important finding in the current study was the reliability of gasping prior to death following carfentanil exposure. All ferrets that died had shown gasping or agonal breathing at some point prior, often immediately prior, to death. Gasping was the most reliable predictor of death. Convulsions, while only observed at higher doses, were not reliably observed in every ferret that received higher doses. The toxic signs and median lethal dose estimates obtained from subcutaneous and IP exposures [17] highlight carfentanil’s potency and can serve as comparisons for future experiments using other routes of exposure (e.g., intravenous injection, ingestion, and inhalation) and treatment with medical countermeasures.

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