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Organoleptic Assessment and Median Lethal Dose Determination of Oral Carfentanil in Rats

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14. ABSTRACT Carfentanil is an incredibly potent and toxic fentanyl analogue that has been targeted as a public health concern. Due to its availability and chemical properties, carfentanil is a potential ingestion hazard, as very small amounts can adulterate a large amount of food or drink. We developed a rat model of voluntary oral ingestion to determine the potential risk carfentanil poses. First, the solubility of carfentanil was assessed in popular consumer beverages: bottled water, apple juice, and 2% milk. Lethality was then assessed by administering carfentanil in bottled water via gavage. A probit model was fit to 24-hour survival data and predicted a median lethal dose of 1.65 mg/kg (95% CI: 0.46 – 2.96 mg/kg; slope: 3.23). Finally, the organoleptic properties (i.e., taste, smell, texture, etc.) were assessed by allowing rats to voluntarily consume 3.0 mL of beverages adulterated at various concentrations. The organoleptics assessment determined that carfentanil was readily detected in water, but was consumed in significantly higher amounts in juice and milk, suggesting these beverages masked its taste or some other sensory property. Buccal absorption also proved to be important, as lethality in the organoleptics assessment was higher than predicted from the lethality assessment utilizing oral gavage. Because rats have more developed chemoreceptive capabilities than humans and are more resistant to opioids, these results suggest that carfentanil may be unwittingly consumed in toxic or even lethal concentrations by humans in a variety of beverages.

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ABSTRACT

Carfentanil is an incredibly potent and toxic fentanyl analogue that has been targeted as a public health concern. Due to its availability and chemical properties, carfentanil is a potential ingestion hazard, as very small amounts can adulterate a large amount of food or drink. We developed a rat model of voluntary oral ingestion to determine the potential risk carfentanil poses. First, the solubility of carfentanil was assessed in popular consumer beverages: bottled water, apple juice, and 2% milk. Lethality was then assessed by administering carfentanil in bottled water via gavage. A probit model was fit to 24-hour survival data and predicted a median lethal dose of 1.65 mg/kg (95% CI: 0.46 – 2.96 mg/kg; slope: 3.23). Finally, the organoleptic properties (i.e., taste, smell, texture, etc.) were assessed by allowing rats to voluntarily consume 3.0 mL of beverages adulterated at various concentrations. The organoleptics assessment determined that carfentanil was readily detected in water, but was consumed in significantly higher amounts in juice and milk, suggesting these beverages masked its taste or some other sensory property. Buccal absorption also proved to be important, as lethality in the organoleptics assessment was higher than predicted from the lethality assessment utilizing oral gavage. Because rats have more developed chemoreceptive capabilities than humans and are more resistant to opioids, these results suggest that carfentanil may be unwittingly consumed in toxic or even lethal concentrations by humans in a variety of beverages.

INTRODUCTION

Carfentanil is a synthetic opioid that is 10,000 times more potent than morphine and 34 times more potent than fentanyl with regards to its analgesic effects [1]. Carfentanil is a tranquilizer for large animals (e.g., moose, elk, and bears) [2] and has no FDA-approved medical application in humans [3]. This opioid has recently garnered the attention of public health officials and first responders due to its availability and misuse as an additive to other drugs [4], such as cocaine, heroin, and benzodiazepines. Opioid abuse has increased in the United States and was responsible for approximately 66% of drug overdose deaths in 2016 [5]. Drug overdose deaths have also been increasing since 1999, and deaths due to synthetic opioids, a class that includes carfentanil and other fentanyl analogues, have more than tripled [5, 6]. The large increase in opioid-related deaths prompted the Drug Enforcement Administration (DEA) to issue a warning about the dangers of carfentanil to first responders and the public in September 2016 [7].

In addition to the public-health concerns, the chemical properties and availability of carfentanil make it a potential terror agent, via ingestion or inhalation. Aerosolized carfentanil and remifentanil (another fentanyl analog) were assumed to be used by the Russian military to incapacitate terrorists and hostages in a Moscow theater in 2002 [8, 9]. Carfentanil may also pose a threat as an ingestion hazard, as it is so potent that a very small amount of carfentanil could adulterate a large amount of food or drink. Several studies have investigated fentanyl delivered via lollipops in an attempt to create a stress-free medication and demonstrated that fentanyl was readily absorbed by the oral mucosa. Analgesia and sedation were reported in human volunteers, and respiratory depression was a common side effect [10-13]. These results, obtained with fentanyl citrate, would also likely extend to carfentanil citrate.

Although there have been several laboratory models of carfentanil exposure using a variety of animal species and routes of exposure [14-17], a paucity of information exists regarding ingested carfentanil. Most of the data sets about oral carfentanil have come from veterinary applications, where carfentanil was delivered in a variety of food or drink items to goats [18], brown bears [19, 20], black bears [21], chimpanzees, capuchin monkeys, gibbons, and bonobos [22].

While the veterinary reports demonstrate that carfentanil is absorbed orally and therefore may be considered an ingestion threat, the species used are not ideally suited as laboratory models. Rats are a more appropriate and expedient laboratory animal model, but there is a lack of information about carfentanil, especially given via the oral route, in this species. To our knowledge, only two studies have used rats to investigate carfentanil: one study determined the median lethal dose (LD_{50}) of intravenous carfentanil in rats to be 3.39 mg/kg [1], and a second study revealed the bioavailability of oral carfentanil as 2.31% [23]. Clearly, further research is needed to better elaborate oral carfentanil toxicity in the rat for use as an ingestion model and to predict human outcomes.

Development of a laboratory animal model of ingestion hazards inherently involves voluntary consumption of potential hazards. While many studies investigating oral hazards use gavage, the intra-esophageal administration of a compound bypasses important oral mucosa, preventing possible intra-oral absorption (i.e., buccal absorption), and ignores the importance of a compound's organoleptic properties. Ingestion hazards that are tasteless and odorless are

more dangerous than those that are readily detectable, with the latter more likely to be rejected prior to the consumption of toxic or lethal amounts. It is beyond dispute that the organoleptic properties (i.e., taste, odor, texture, and other physical properties) of these chemicals are understudied in toxicology. Because rats will eat many of the same foods that humans eat, actual food and/or drink items of interest may be used and adulterated to determine realistic oral-ingestion threats. Likewise, rats are neophobic [24] and will tend to refuse new items, making them a conservative model when testing for threat organoleptics. Rats also have chemoreceptive capabilities superior to humans, so any compound a rat consumes in toxic concentrations would likely be consumed by humans as well. In this study, we leveraged the rat's chemoreceptive capabilities to test the organoleptic properties of carfentanil in several beverages popular in the U.S.: bottled water, apple juice, and 2% milk. By assessing the organoleptic properties in addition to the resulting toxicity, we were able to further profile carfentanil's toxicity in a rat model while also developing a comprehensive threat assessment of carfentanil as an oral-ingestion hazard.

METHODS

Chemicals and Matrices

Carfentanil citrate (2-hydroxypropane-1,2,3-tricarboxylic acid; methyl 1-(2-phenylethyl)-4-(N-propanoylanilino)piperidine-4-carboxylate; approximately 98% purity) was obtained from the U.S. Army CCDC Chemical Biological Center and stored protected from light at room temperature. All handling of carfentanil citrate prior to being placed into solution occurred within the confines of a certified chemical fume hood.

Aquafina® purified drinking water (16.9 oz, 500 mL; 24 pack), Mott's® 100% apple juice (8 oz, 237 mL; 6 pack), and Cloverland® 2% milk (1 quart, 946 mL; single bottle) were purchased from local vendors. The water and apple juice were purchased and stored at room temperature for up to several weeks prior to being placed in a refrigerator at least 24 hours prior to use. Milk was purchased at the beginning of every week and kept refrigerated at approximately 4° C.

Subjects

One hundred twenty (120) male Sprague-Dawley rats (SAS SD 400) were obtained from Charles River Laboratories (Wilmington, MA, USA). Thirty (30) rats were assigned to the median lethal dose determination, and 90 rats were assigned to the organoleptics assessment. All rats weighed between 226-250 g at the time of shipping and were allowed five days (under group housing) to acclimate to our facility. All subjects were housed individually thereafter in a vivarium with free access to water under a 12-hour light/dark cycle (lights on at 0600). All rats had free access to food and water during acclimation, after which water regulation was implemented and maintained for the remainder of the study (food remained freely available). Water regulation was implemented by pulling the cages of the rats outward several inches, removing the ability to drink from the water valve. When water was made available, the cages were pushed back several inches until the water valve was inserted into the home cage. Water access was limited to 2 hours per day (typically from 1230 to 1430) and occurred at least one hour after the organoleptics assessment training.

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, the Public Health Service Policy on Humane 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.

Solubility Determination

Carfentanil solubility was assessed in room-temperature (21° C) water as well as refrigerated (4° C) water, juice, and milk. Each assessment began with a known amount of carfentanil (1.47 – 4.67 mg; *M* = 3.60, *SD* = 0.90; larger amounts were used for milk compared to water and juice) placed into a vial followed by 0.1 – 9.7 mL of a beverage (selected to approximate 90% of the expected solubility). An incremental volume of 0.025 – 0.25 mL (based on the expected solubility; 0.025 and 0.035 mL was used for water, 0.025 mL was used for juice, and 0.25 was used for milk) was then repeatedly added until solubility was achieved, and the final concentration was recorded. Mechanical agitation (5-second duration) with a Vortex-Genie 2 laboratory mixer (Daigger Scientific Inc., Vernon Hills, IL) followed each incremental addition, and a 10-second partial submersion in an ice-water bath followed every third incremental addition to keep the solution at the appropriate temperature. This assessment was conducted three times for each beverage.

Median Lethal Dose Determination

A stagewise, adaptive dosing design [25-27] was used to determine the median lethal dose (LD_{50}) of carfentanil in 21° C water. Doses for the first stage were selected based on the available literature [1, 23]. Doses for the second and all subsequent stages were based on 24-hour lethality observed from the previous stage(s). Doses were administered via gavage in 2.5 – 3.0 mL of 21° C water, and all subjects were observed continuously for the first hour and then checked hourly thereafter until 5 hours post-exposure. A final observation occurred at 24 hours post-exposure, where survivors were humanely euthanized. Doses were selected such that the entire range of lethality (0% to 100%) was observed. Probit models using maximum likelihood estimates were fitted to the combined data for all stages.

Organoleptics Assessment

The organoleptics assessment occurred in a polycarbonate rodent cage (45.7” X 24.1” X 20.3”) with an air-filtered lid. A polycarbonate insert was placed into the bottom of the cage that had a cutout for a 5.72 cm diameter smooth tempered glass condiment dish to rest in. The glass dish was harder for the rats to tip over or remove when placed in the cutout. All beverages used in the organoleptics assessment were refrigerated (4° C). Training for the assessment occurred for 8 days prior to exposure to the adulterated beverages. The first two training days allowed the rats 10 min to consume up to 10 mL. The following three days gave the rats 5 min to consume 5 mL, and the final two training days were 5 min to consume 3 mL. Rats had to consume at least 2.5 mL on the day prior to exposure to be included in the analysis and all rats met the criterion.

The beverages were adulterated with carfentanil prior to being distributed to the glass dishes on the day of exposure. The volume of the adulterated beverage was 3.0 mL. The concentration of the adulterated beverage was equivalent to the LD_{50}, LD_{25}, LD_{10}, LD_{01}, or ½ LD_{01}, assuming a 300-gram rat consumed the entirety of the 3.0 mL adulterated beverage. The first concentration used in this assessment was the LD_{50} equivalent for all beverages. Subsequent concentrations varied according to the consumption observed with the previous concentration(s) and were allowed to vary between beverages. This assessment was repeated with new concentrations until at least 9 out of 10 rats were consuming at least 2.5 mL of the adulterated beverage.

Statistical Analysis

The median lethal dose estimate and associated 95% confidence interval were obtained using the methods of Feder et al. [25-27] and the SAS PROBSEP program. After each stage, probit dose response models using maximum likelihood methods were fitted to the combined data from all stages. A stopping criterion was used and defined as (95% upper confidence interval of the LD_{50} – 95% lower confidence interval of the LD_{50}) / (2× LD_{50}) < 0.40.

If the stopping criteria were not met and the maximum number of animals was reached, no further animals were used. The estimated LD_{50} at that point was accepted as adequate.

RESULTS

Solubility Determination

The solubility of carfentanil was assessed in multiple beverages, both room-temperature and refrigerated, as shown in Table 1. Although moderately soluble in room-temperature water, refrigeration significantly decreased solubility. The dissolved solids and other physical attributes of the juice and milk apparently decreased solubility as well.

Table 1

Solubility of carfentanil citrate in bottled water, apple juice, and 2% milk. Solubility was assessed three times (indicated by the numbered headings) per beverage, including an additional assessment with room-temperature (21° C) water. All solubility data are presented as mg/ml.

Beverage	Temp	1	2	3	Mean	SD
Water	21° C	3.27	3.31	2.90	3.16	0.22
Water	4° C	1.54	1.40	1.40	1.45	0.08
Juice	4° C	0.90	0.96	1.00	0.95	0.05
Milk	4° C	0.26	0.24	0.23	0.24	0.01

Median Lethal Dose Determination

A probit model was fit to 24-hour survival data and predicted a median lethal dose of 1.65 mg/kg (95% CI: 0.46 – 2.96 mg/kg; slope: 3.23). The combined probit function and the observed survival proportions are shown in Figure 1. Subjects were continuously observed for the first hour following exposure, and the general progression of toxic signs was noted. Lethargy and freezing were the most common initial signs, though the highest carfentanil dose assessed (29.80 mg/kg) did not produce overt freezing due to the rapid onset of muscle rigidity. In general, muscle rigidity often followed lethargy and freezing, with the severity of the rigidity varying, increasing with dose. Lower carfentanil doses produced mild muscle rigidity, and higher doses produced such profound muscle rigidity that rats were completely stationary, even when picked up or moved. Straub tail was also common during periods of muscle rigidity and was observed across a wide range of doses (1.26 – 29.80 mg/kg). Breathing changes often followed or simultaneously occurred with muscle rigidity. Most breathing changes were characterized by decreased depth and rate; irregularity was also observed. Finally, high doses of carfentanil produced periods of prolonged apnea, which often led to death.

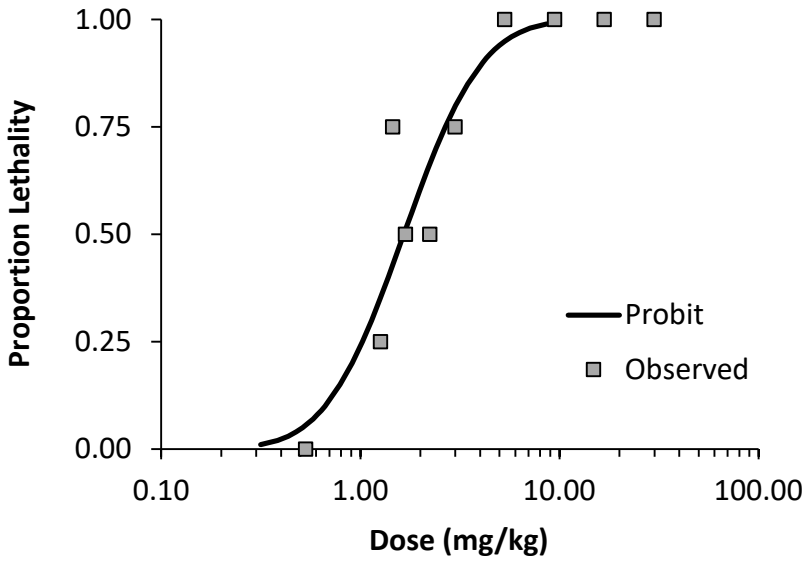

Figure 1. Probit model of 24-hour survival as a function of carfentanil dose (mg/kg). Observed survival rates at each dose are shown as gray squares, and the fitted model is shown as a black line. The estimated median lethal dose was 1.65 mg/kg

Organoleptics Assessment

Rats were given the opportunity to voluntarily consume water, apple juice, and milk adulterated with carfentanil at various concentrations, shown in Figure 2. If the volume consumed was at least 2.5 mL, the adulterated liquid was scored as “accepted” and considered to be generally palatable. The number of rats that accepted the adulterated beverages is shown in Table 2. The LD_{50}-equivalent concentration was the first to be assessed, and matrix-dependent acceptance was observed. Five of 10 rats drank over 2.5 mL when carfentanil was placed in milk, whereas only 2 out of 10 rats accepted the adulterated juice. None of the rats accepted the adulterated water, and the amounts consumed were significantly lower than for the other two beverages. Based on these results, water was further assessed at the LD_{10}-equivalent concentration, and again no rats consumed at least 2.5 mL. This concentration was lowered to the LD_{01} equivalent, and 7 out of 10 rats accepted the adulterated water; all 10 rats accepted the adulterated water when the concentration was subsequently halved.

Juice and milk appeared to mask the taste of carfentanil, so the LD_{25}-equivalent concentration was the next to be assessed (unlike water, which was assessed with the LD_{10}-equivalent concentration). Eight out of 10 rats accepted the adulterated juice, and 9 out of 10 rats accepted the adulterated milk. The final concentration assessed for juice was the LD_{10} equivalent, and 9 out of 10 rats consumed at least 2.5 mL. The actual amounts consumed (not scored as “accept” or “reject”) are shown in Figure 2, and they clearly demonstrate a masking effect of juice and milk compared to water. Rats consumed larger volumes of juice and milk at similar or higher concentrations compared to water.

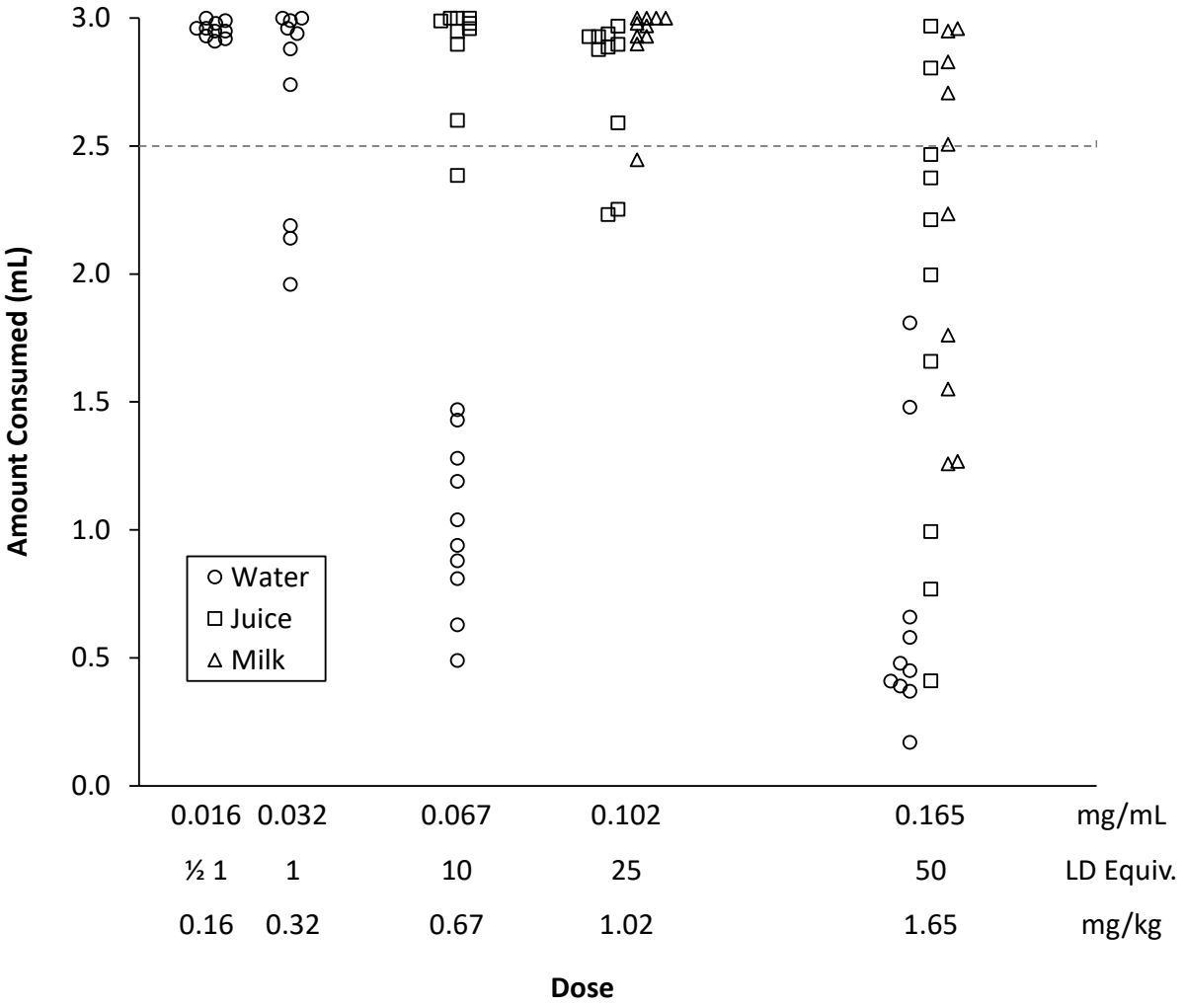

Figure 2. Amount of adulterated beverage consumed for all concentrations assessed as a function of beverage. Water is shown as circles, juice is shown as squares, and milk is shown as triangles. Each data point represents an individual subject's volume consumed. The gray, dashed line represents the 2.5 mL threshold to be counted as "accepted."

Table 2

The number of rats that "accepted" (i.e., consumed at least 2.5 mL) the adulterated beverages as a function of concentration (shown as LD equivalents).

	Concentration (LD Equivalent)				
Beverage	**½ LD_{01}**	**LD_{01}**	**LD_{10}**	**LD_{25}**	**LD_{50}**
Water	10/10	7/10	0/10	-	0/10
Juice	-	-	9/10	8/10	2/10
Milk	-	-	-	9/10	5/10

As shown in Table 3, 24-hour lethality following voluntary consumption of carfentanil-adulterated beverages was often higher than estimated based upon the median lethal dose determination using gavage. Although no rats consumed more than 2.5 ml, seven rats out of 10 died after consuming the LD_{50}-equivalent concentration in water. Six out of 10 rats died when they consumed the same concentration in juice and milk. The expected lethality was approximately 5 out of 10 rats, assuming the rats drank the entirety of the 3.0 mL solution. However, many of the rats drank less (some significantly so) than 3.0 mL, so lethality should have been lower as well. Higher-than-expected rates of lethality were also observed when rats consumed the LD_{10}- and LD_{01}-equivalent concentrations in water.

Table 3

The number of rats that died within 24 hours of consuming an adulterated beverage as a function of concentration (shown as LD equivalents).

	Concentration (LD Equivalent)				
Beverage	**½ LD_{01}**	**LD_{01}**	**LD_{10}**	**LD_{25}**	**LD_{50}**
Water	0/10	2/10	3/10	-	7/10
Juice	-	-	1/10	1/10	6/10
Milk	-	-	-	0/10	6/10

Changes in body weight 24 hours after exposure were also recorded as a secondary measure of intoxication (or recovery). The body weight changes are shown in Figure 3 for any rat that survived to 24 hours. As expected, higher doses of carfentanil produced more intoxication, shown here as negative body weight change (i.e., weight loss). The ½ LD_{01}-equivalent concentration failed to produce long-term toxicity, and all rats gained an appropriate amount of weight overnight, indicating that they recovered enough to eat and drink during the 2-hour window that water was made available. Interestingly, the LD_{10}-equivalent concentration produced markedly different weight changes for animals that consumed carfentanil in water and juice. Rats typically consumed less carfentanil when in water compared to juice, but water survivors were often more intoxicated, shown here by the overnight body weight loss.

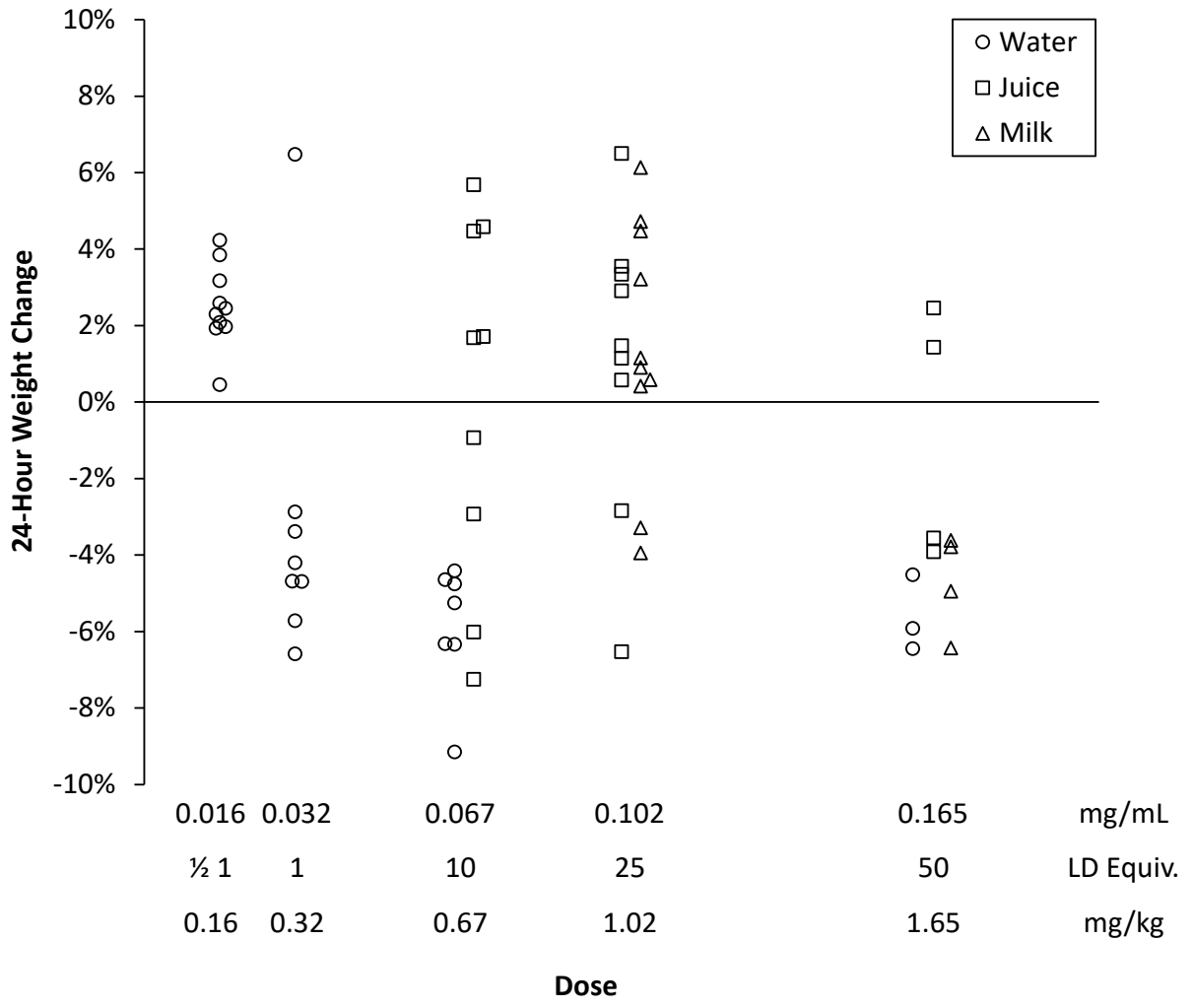

Figure 3. Change in body weight 24 hours after consumption of adulterated beverages for all concentrations assessed. Water is shown as circles, juice is shown as squares, and milk is shown as triangles. Each data point represents an individual subject that survived to 24 hours.

DISCUSSION

A three-phase approach was used in the current experiment to assess carfentanil's potential as a food or drink adulterant. First, the solubility of carfentanil in the refrigerated beverages was assessed. Solubility was decreased by refrigeration and also varied by beverage type (Table 1). Solubility was highest for water, followed by juice, and then finally milk. The solubility of carfentanil in refrigerated milk is poor, but is still high enough to be toxic or lethal when consumed.

The toxicity of carfentanil delivered via gavage was then assessed, and the median lethal dose (LD_{50}) was estimated to be 1.65 mg/kg. This result was surprising, as the estimated LD_{50} of IV carfentanil was reported as 3.39 mg/kg [1]. However, that particular study is bereft of details about how the LD_{50} assessment was performed, and there is no guarantee that appropriate

methodological considerations, such as subject numbers and dose selections, were made. Second, the oral availability of carfentanil was estimated to be 2.31% of an equivalent IV dose [23], suggesting that the oral LD_{50} would be significantly higher than the IV estimate. Taken together, our estimate is lower than predicted by these two sources, but the lethality observed across a range of doses concur with the estimated LD_{50}. The associated 95% confidence intervals are also sufficiently similar to the median lethal dose, which suggests that the estimate obtained in the current experiment is supported by the data. Likewise, the data obtained in the organoleptics assessment also suggest that the oral LD_{50} is lower than the 3.39 mg/kg IV estimate suggested elsewhere [1].

The organoleptics assessment occurred after the median lethal dose determination. Here, the three refrigerated beverages were adulterated with carfentanil at various concentrations corresponding to estimated doses from the probit function. The LD_{50}-equivalent concentration was the first to be assessed and a clear matrix-dependent effect was observed, as shown in Figure 2. Carfentanil was readily detected and rejected in water, rejected less often in juice, and then rejected least often in milk. This suggested that juice and milk afforded some "masking" of the carfentanil organoleptics. These beverages likely masked carfentanil's taste, but this is unconfirmed and the beverages could also be masking the smell, texture, and/or some other property. Based on the LD_{50}-equivalent results, different concentrations were assessed with different beverages. Juice and milk were accepted by the majority of rats when given at an LD_{25}-equivalent concentration or an LD_{10}-equivalent concentration. Once again, milk was accepted at higher rates than juice. Water, on the other hand, was again rejected at an LD_{10}-equivalent concentration, whereas adulterated juice was accepted by 9 out of 10 rats at the same concentration. Rats only began accepting adulterated water when it was at the LD_{01}-equivalent concentration, and all rats accepted the adulterated water when it was given at one half of the LD_{01}-equivalent concentration.

Intoxication following carfentanil ingestion was categorized in two ways: 24-hour lethality and weight change. In the animals that survived, weight change served as a good indicator the duration of intoxication, as animals that recover sooner are more likely to consume food and gain weight overnight. This is particularly true of the current experiment, as water access is timed and occurs relatively soon after carfentanil ingestion. Any rats that were intoxicated for extended periods may fail to drink their daily allotment of water. In general, lower doses of carfentanil produced less intoxication and body weights were typically higher the following day (Figure 3). Lethality was highest in the rats that ingested the LD_{50}-equivalent concentration, even though the majority of the rats consumed less than the 3.0 mL of the adulterated beverage. The LD_{50}-equivalent was computed based on the rats drinking 3.0 mL, so the poor consumption should have translated into lower rates of lethality. This was also observed with the LD_{10}-equivalent and LD_{01}-equivalent concentrations: significantly more rats died than expected. These results suggest that the absorption of carfentanil through the oral mucosa is significant, which has also been observed in humans and other animals. Less sedation and analgesia was observed in human volunteers that rapidly consumed a fentanyl-adulterated lollipop compared to volunteers that allowed the lollipop to dissolve in the mouth [13]. Chimpanzees that consumed carfentanil in grapes and oranges were far less intoxicated than those that consumed it in marshmallow crème, presumably because of the increased contact

time with the oral mucosa [22]. Bears that took longer to eat honey adulterated with carfentanil reached sternal recumbency faster, suggesting buccal absorption is important [21] and carfentanil given orally in goats was also primarily absorbed in the oral mucosa as opposed to the GI tract [18].

All of these studies demonstrate that the oral absorption of carfentanil (and likely other fentanyl analogs) is important and a distinction needs to be made between different methods of oral delivery/ingestion. For example, one of only two studies using carfentanil in rats suggested that the bioavailability of carfentanil when given PO was 2.31% [23]. This study fails to give details about the PO administration and it is assumed to be via gavage, meaning that the carfentanil was absorbed in the GI tract. This 2.31% bioavailability is ignoring the importance of buccal absorption and does not accurately reflect the toxicity and danger that carfentanil poses when ingested. In addition to this, there is evidence to suggest that while carfentanil is easily detected in water, juice and milk can mask the taste and rats will consume significantly higher concentrations (and higher volumes) of the adulterated beverage. Rats have more developed chemoreceptive capabilities than humans and are more resistant to opioids, so these results suggest that carfentanil may be consumed in toxic or lethal amounts by humans in a variety of beverages.

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