Journal of the American Association for Laboratory Animal Science
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Vol 64, No 3
May 2025
Pages 514–519

A Single-Dose Pharmacokinetic Study of Metronidazole Administered to Gottingen Minipigs (*Sus scrofa*) by Oral Gavage or Voluntary Oral Dosing

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Oral gavage (OG) dosing can be stressful to pigs and is associated with the risk of complications. To evaluate a potential refinement, we compared the pharmacokinetics of a drug with a known aversive taste (metronidazole) administered orally to 6 Gottingen minipigs with voluntary cooperation of the animal (voluntary oral [VO]) to 6 Gottingen minipigs dosed by OG. Blood was collected predose and at 0.5, 1, 2, 4, 8, and 24 h postdose and analyzed for drug concentration. Pharmacokinetic parameters for metronidazole were calculated, including C_{max}, T_{max}, AUC (from 0 to 24 h), and the ratios of AUC and C_{max} between 2 dosing methods (AUC OG/VO ratio and C_{max} OG/VO ratio, respectively). The time required to dose (dosing interval) and animal and staff acceptance of the dosing procedures were also assessed. All animals were dosed successfully, but one animal in each group was noted to have dosing difficulty. Mean dosing interval for OG dosing was greater than for VO (2.6 ± 0.24 min SD compared with 1.6 ± 0.36 min, respectively). VO dosing required fewer handlers, appeared to be less stressful to the animals, and was reported to be more ergonomically favorable than OG dosing. There were no significant differences in exposure including C_{max}, T_{max}, and AUC between OG and VO dosing. The OG/VO ratio was 1.27 for AUC and 1.25 for C_{max}. Both animals with difficulty during dosing had pharmacokinetically inconsistent concentration–time profiles when compared with all other animals. These apparent differences were within the expected variability seen in pharmacokinetic studies. VO dosing may be a potential refinement for a single-dose pharmacokinetic study of an aversive tasting test material to minipigs.

Abbreviations and Acronyms: OG, oral gavage; QC, quality control; VO, voluntary oral

DOI: 10.30802/AALAS-JAALAS-25-021

Introduction

The minipig is an established animal model for nonclinical studies, with many anatomic and physiologic similarities to humans.[1] One challenge with pig models is that they are relatively intolerant of oral gavage (OG) dosing.[2,3] The procedure can be stressful and carries a risk of injury, which can affect animal welfare as well as study data.[2,3] However, OG is typically used because it ensures that the exact amount of the test article is administered to the stomach.[4] A basic tenet of animal welfare regulations and standards is to use refinement alternatives to reduce stress whenever possible during conduct of an animal study.[5,6] Voluntary oral (VO) dosing is commonly employed in companion animal pharmaceutical development; however, significant preliminary work is required to assess compatibility and palatability to ensure dosing success and consistent exposure.[7] In rodents a more generic approach of first habituating the animal to a positive food reward and then substituting the dosing material has been employed with success.[8–11] This approach is desirable in a nonclinical testing environment so that instead of having to develop dosing procedures for each test material, a standardized training procedure can be developed with potential application for numerous test materials. It is particularly suitable for use in pigs because of their well-known food motivation; however, a literature search did not indicate that this method had been compared with OG dosing for comparability of pharmacokinetic parameters.

A test article with an aversive taste was selected to 'pressure test' this method to see whether the aversiveness could be overcome with the positive reinforcement reward. Metronidazole is known to have poor palatability in some animals[12] and in humans,[13] with bitterness being the flavor it is known for anecdotally among veterinary professionals. While palatability of metronidazole has not been studied in pigs, there are known similarities in taste sensitivity between humans and pigs, including response to bitter flavor.[14] In this study we assessed the exposure of metronidazole administered by OG with VO dosing. We also evaluated practical aspects of the 2 dosing methods, such as animal acceptance, staff perception, and time for dose administration (dosing interval).

Materials and Methods

Animal subjects. All procedures were performed under a protocol approved by the IACUC in an AAALAC-accredited, USDA-registered vivarium. Twelve female Gottingen minipigs (Marshall BioResources, North Rose, NY) ~8 mo of age were

Submitted: 20 Jan 2025. Revision requested: 19 Feb 2025. Accepted: 30 Mar 2025.
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used for this study. The microbiologic status of the animals was not determined and was considered conventional. Group size was determined based on historical experience with large animal pharmacokinetic studies, as no data on the pharmacokinetics of metronidazole administered by OG to swine were available for sample size power analysis. Room temperature was 73.6 °F (23.1 °C) mean (range, 70.4 °F to 77.1 °F [21.3 to 25.1 °C]), relative humidity was 52.5% mean (range, 39.4% to 81.1%), and the room air exchange rate was 10 to 15 changes per hour. Overhead fluorescent lighting was provided on a 12-h light/12-h dark cycle. Animals were socially housed (pair or trio) in stainless steel runs with elevated, plastic-coated, perforated floors, with durable, nonedible, manipulanda provided for enrichment. They were separated temporarily for dosing and blood collection. Municipal water was provided ad libitum and was tested annually for contaminants listed in the U.S. Environmental Protection Agency primary drinking water standard. Animals were fed ~300 to 400 g of LabDiet 5K99/certified porcine grower/maintenance diet (PMI International, Richmond, IN) divided into twice daily feedings. Approximately ¼ cup of a lightly sweetened dry breakfast cereal (Cinnamon Toast Crunch, Gordon Food Service, Wyoming, MI) was provided once daily for enrichment. Following blood collection, small amounts of dried cantaloupe were offered for enrichment. Animals were returned to the training colony at the end of the study.

Study design. Animals were randomly assigned identification numbers on receipt. They were selected for study based on physical examination (no abnormal findings) and assigned to a dosing group (n = 6/group) to balance the body weight range between groups (OG, 12.9 to 21.9 kg; VO, 14.4 to 21.4 kg). No specific procedures were employed to avoid confounding variables for sequence of procedure or location of cage. Study personnel who provided observations on dosing acceptance were not blinded to treatment, as the method of dosing could not be concealed. Beginning 2 wk prior to dosing, animals in the VO group were trained on the procedure once daily, except on weekends, for ~2 to 3 min by being introduced to a dosing syringe with 20 mL of white grape juice (Harvest Valley Fruit Juice, Gordon Food Service, Wyoming, MI) instilled into the oral cavity for 2 d, subsequently replaced with tap water delivered via a syringe, followed by ~5 to 10 mL of juice from a squeeze bottle as a reward for consuming the liquid from the syringe. The OG group was habituated to the dosing procedure by being placed in a restraint sling and orally gavaged with 10 mL of tap water on 3 occasions beginning 1 wk prior to dosing. Food rewards (breakfast cereal or dried cantaloupe, as described for enrichment) were provided following the procedure for procedural desensitization.

Animals in both groups were dosed with metronidazole (metronidazole tablets, USP, 250 mg, Viona Pharmaceuticals, Cranford, NJ) crushed and added to 0.5% carboxymethylcellulose in deionized water, mixed until uniform for a final dose volume of 5 mL/kg and a final dosage of 50 mg/kg metronidazole for each animal. Carboxymethylcellulose was selected because it is a very common vehicle used in nonclinical animal studies and represents a likely condition under which VO dosing may be applied. OG was performed with the animal in sling restraint using an oral speculum and a 16-in (40.6-cm), 18-Fr tube (Covidien, Dublin, Ireland). The tube was inserted into the caudal pharynx and advanced as the animal swallowed. A syringe was connected and the plunger was drawn back to ensure proper placement (vacuum or stomach contents indicate the tube is not in an airway). The entire dose volume was administered, followed by 10 mL of tap water; the tube was kinked to prevent aspiration and then withdrawn. VO dosing was performed as described for the training procedure, with the test material delivered via syringe and the grape juice reward provided intermittently throughout dosing to reward the behavior of swallowing the test material (approximately 5 to 10 mL of juice up to 3 times during dosing).

Blood collection intervals were selected based on a review of concentration–time data and pharmacokinetic parameters of metronidazole in other species. Blood was collected for bioanalysis beginning prior to dosing, and then at 0.5, 1, 2, 4, 8, and 24 h postdose from the jugular vein or cranial vena cava. At each timepoint, 0.5 mL of blood was collected into a tube with K_2EDTA and placed on wet ice. The samples were then centrifuged and the plasma removed, transferred to a tube on dry ice, and then stored at −60 °C to −90 °C until analysis.

Bioanalysis. Calibration standards, ranging from 1.00 ng/mL lower limit of quantitation to 1000 ng/mL upper limit of quantitation of metronidazole (USP, Rockville, MD), were prepared in pig plasma with K_2EDTA (BioIVT, Hicksville, NY). Calibration standards were analyzed in duplicate from a single well for each concentration, and the responses were regressed against the nominal concentrations using a quadratic curve fit with 1/×2 weighting. The analytical runs were accepted as the back-calculated concentrations of at least 70% of the calibration standards and were within 20.0% of their theoretical concentrations (within ±25.0% at the lower limit of quantitation).

Six replicates of quality control (QC) low, middle, and high samples were included in the first run to qualify the assay. Four replicates of QC low, middle, and high samples were analyzed in each subsequent analytical run. Three replicates of dilution QC samples were added to each run with diluted samples. The analytical runs were accepted with an accuracy of ≥66.67% of the QC samples and ≥50% per concentration levels were within the criterion range of 80.0% to 120.0%.

Each 50-µL aliquot of standard, QC, or study sample was mixed with 50 µL of working internal standard solution (100 ng/mL metronidazole-d4 [Cayman Chemical, Ann Arbor, MI] in 4 g/dL BSA [Sigma-Aldrich, St. Louis, MO]) and 400 µL of methanol/acetonitrile (50:50, v/v) (VWR Chemicals BDH, Radnor, PA and Sigma-Aldrich, St. Louis, MO, respectively). The samples were vortexed and centrifuged. A 200-µL aliquot of the resulting supernatant was transferred to a clean 96-well 1-mL autosampler plate, evaporated under nitrogen, and reconstituted with 100 µL of acetonitrile/water/formic acid (EMD Millipore, Burlington, MA) (10:90:0.1, v/v/v). An aliquot was injected onto a Nexera X2 UPLC-MS/MS for analysis (Shimadzu, Kyoto, Japan and SCIEX, Framingham, MA) using an XSelect HSS-T3 column, 100 Å, 2.1×50 mm (2.5 µm particle size) (Waters Corporation, Milford, MA) with an isocratic flow consisting of water/formic acid (100:0.1, v/v) and acetonitrile/formic acid (100:0.1, v/v) at a flow rate of 0.3500 mL/min. The analyte and internal standard were detected using a SCIEX API 5000 triple quadrupole LC-MS/MS system equipped with a TurboIonSpray electrospray ionization source operated in the positive ion mode. Multiple reaction monitoring transitions m/z 172.2 → 128.0 at the retention time of 0.9 to 1.1 min were used to monitor metronidazole and m/z 176.0 → 128.0 at the retention time of 0.9 to 1.1 min were used to monitor metronidazole-d4. All LC-MS/MS data were acquired and processed using Analyst software version 1.7.2 (SCIEX, Framingham, MA) and quantitated using Watson LIMS software (Thermo Fisher Scientific, Waltham, MA).

Pharmacokinetic analysis. A noncompartmental analysis was used for parameter estimation using Phoenix pharmacokinetic software, version 8.3 (Certara, Radnor, PA). For metronidazole

in plasma, the extravascular model was used for parameter estimation. All parameters were generated from metronidazole individual concentrations in plasma collected during the study. Parameters were estimated using nominal dose levels and nominal sampling times relative to the dose administration. Plasma concentration values obtained at the predose time point were used as the concentration at time 0. Concentration values reported as below the limit of quantitation (<1 ng/mL) were treated as 0. The area under the concentration compared with the time curve (AUC) was calculated using the linear trapezoidal method with linear interpolation for all profiles with at least 3 consecutive quantifiable concentrations. $AUC_{0–24\,h}$ was reported when the AUC%extrap was ≤25% of the total area.

Statistical analysis. The time the dose was administered was recorded, and a dosing interval for each method was calculated by subtracting the time of each dose from the previous time of dose. This number is the dosing interval, which is an estimation of the time required to perform the procedure. Because it is a calculated value, there are only 5 intervals for the 6 test subjects. Differences between mean dosing intervals were tested for normal distribution using the Shapiro-Wilk test, and the difference between means was analyzed using a Student 2-tailed *t* test with a Welch correction. The C_{max} of metronidazole, T_{max} of metronidazole, and AUC for each group ($n = 6$/group) were analyzed for normal distribution, and then differences between groups were analyzed using the Kolmogorov–Smirnov test. All statistics were performed using Prism version 9.4.0 (GraphPad Software, Boston, MA).

Results

One animal in the VO group did not have any blood collected at the 2 and 24 h timepoints due to fractious behavior, and these data were excluded from the analysis. Anecdotally, the trainer indicated this animal had the most variable behavior of the group during the training sessions. One animal each in the VO and OG groups was reported with difficulty dosing. However, the full dose was administered to these animals, so their data were included in the subsequent analysis. Three to 4 handlers were required for OG dosing each animal whereas only 2 were needed for VO dosing. Handlers commented that VO dosing appeared to be less stressful to the animals and was more ergonomic than OG dosing.

The dosing interval for OG was significantly greater than for VO (mean of 2.6 min OG compared with 1.6 min VO, $P \leq 0.05$, $n = 6$) (Figure 1). Technical staff reported that the VO dosing procedure went very smoothly and that animals and staff seemed less stressed than with the OG dosing.

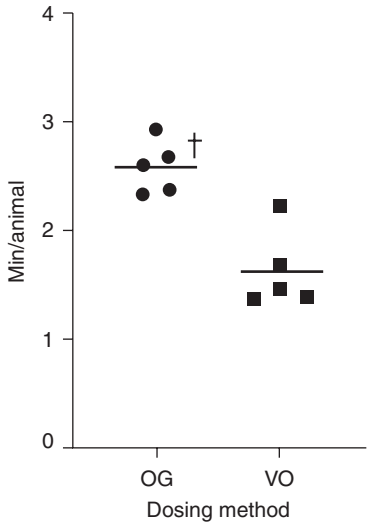

Figure 1. Dosing interval for oral gavage compared with voluntary oral dosing of Gottingen minipigs. The time in minutes is shown between sequential dosing of animal test subjects for 2 methods of dosing (OG, oral gavage; VO, voluntary oral); $n = 5$ intervals between 6 animals. The horizontal bar represents the group mean. †, $P \leq 0.01$

There were no statistical differences in C_{max}, T_{max}, and AUC between groups ($P > 0.05$, $n = 6$) (Figure 2). To assess similarities or differences between the differential routes of administration for the OG and VO groups, a comparison of pharmacokinetic parameters C_{max} and AUC (the ratio of C_{max}/AUC values for OG/VG) were determined. The OG/VO ratio was 1.27 for AUC and was 1.25 for C_{max}. The variance from unity (ratio = 1) was within the generally acceptable range of no difference (0.8 to 1.25) as per bioequivalence measures. Individual animal pharmacokinetic curves by group are presented in Figure 3 to visualize data variability.

Discussion

The results of this study indicate that the dosing interval is significantly reduced while animal acceptance, personnel ease of use, and drug exposure are comparable to slightly improved for VO dosing compared with OG dosing. Although there was not a statistically significant difference between the calculated pharmacokinetic parameters for the 2 dosing methods, there

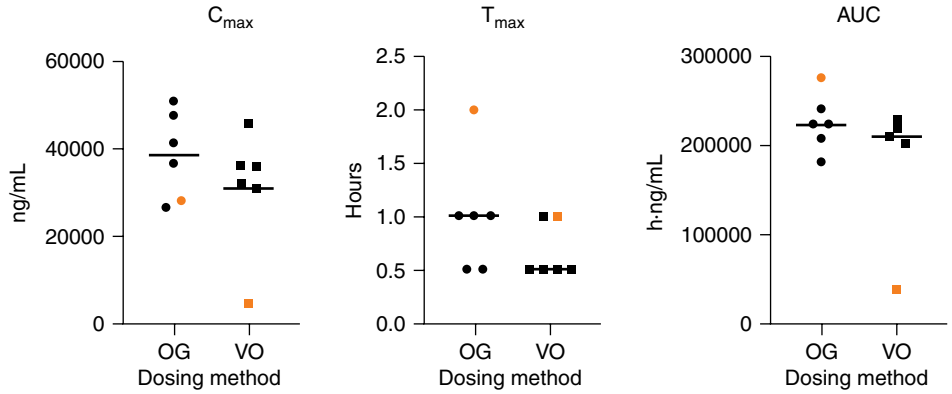

Figure 2. Pharmacokinetic parameters for metronidazole administered by oral gavage or voluntary oral dosing to Gottingen minipigs. Pharmacokinetic parameters are shown for metronidazole in minipigs dosed by 2 different methods (OG, oral gavage; VO, voluntary oral). The AUC shows 0 to 24 h. $n = 6$. The horizontal bar represents the group mean. Round markers indicate OG dosing. Square markers indicate VO dosing. Orange markers represent the individual animal in each group that experienced difficulty with dosing. There were no significant differences between means for C_{max}, T_{max}, or AUC ($P > 0.05$)

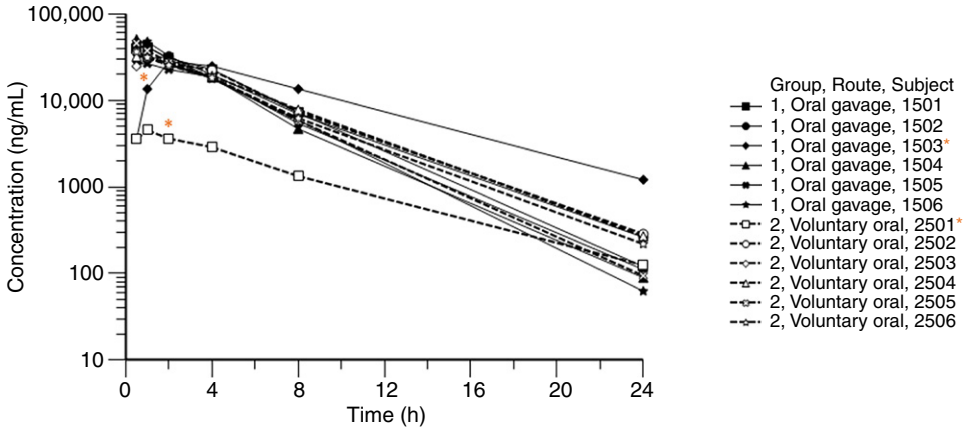

Figure 3. Individual pharmacokinetic curves for Gottingen minipigs dosed with metronidazole by oral gavage or voluntary oral dosing. Concentrations are shown of serum metronidazole at blood collection timepoints for individual animals dosed by 2 different methods. Solid markers indicate oral gavage dosing. Open markers indicate voluntary oral dosing. An asterisk indicates pharmacokinetically inconsistent concentration–time curves for a single animal in each group. Both animals had documented dosing difficulty.

appeared to be a trend to higher exposure in the OG group compared with the VO dosing group. This trend is driven by the 2 pharmacokinetically inconsistent time profiles associated with dosing difficulty. If these animals are excluded, the OG/VO AUC ratio is 1.00 and the ratio for C_{max} is 1.13.

One of the primary advantages of VO dosing over OG dosing is that it utilizes training and positive reinforcement instead of physical restraint, a known stressor.[4] This advantage seemed to be evident in this study based on the feedback from personnel on animal acceptance and staff perceptions of the procedure. However, we did not specifically assess measures of stress, such as stress hormones or a detailed behavioral analysis, so these observations can only be considered anecdotal. Another advantage of the VO dosing method is that there should be minimal risk of misdosing or dosing injury that could occur as a complication with OG dosing. We did not see complications from either dosing method in this study, but it is possible additional complications could be seen when evaluated on a larger scale.

There are some limitations of this study that need to be considered before applying these results to novel situations. We observed a decreased time for dosing and the need for fewer people to perform VO compared with OG dosing. However, the voluntary method used a longer lead time to train the animal on the procedure (5 d a week for 2 wk) compared with habituation to the restraint sling and gavage (3 sessions in 1 wk). Although we did not document training uptake time, the trainer reported that most animals were trained to VO dosing after the second session, suggesting that the lead time for training could be shortened to be comparable to, or even shorter than, that required for OG dosing. It is also noteworthy that similar to the dosing procedure, the training procedure itself required fewer people for less time for VO compared with OG dosing.

One of the historical objections to voluntary dosing procedures has been the risk of incomplete dosing or a prolonged dosing interval that would affect exposure and other pharmacokinetic parameters.[4] We did not observe that to be a significant factor in this study, except for the single animal in the VO group. Because there was also a single animal in the OG group that resisted dosing, the 2 methods were comparable in this regard. The observations of difficulty dosing aligned directly with the pharmacokinetics data, suggesting that on a pharmacokinetic study, it would be possible to exclude data from animals that had incomplete dosing due to procedural failure to distinguish it from actual variability in pharmacokinetics. Note, however, that there could be other factors that would affect the feasibility of VO dosing that were not assessed within this study. We specifically selected a test article that is known to be highly aversive in humans and was thought to be likely to be aversive in pigs due to a bitter taste.[14] It is possible that a novel test article could present a flavor that cannot be overcome by the training procedure and positive reward. We also only dosed the animal with test article once. Anecdotal data from early methods development work indicated that repeated dosing with metronidazole did not result in acquired intolerance of the dosing procedure, but it is possible that a novel test article with a different flavor might cause aversiveness that cannot be overcome with the training and reward procedure. Also, we have observed anecdotally that animals can develop aversion to any dosing procedure if the test article itself induces local or systemic discomfort. It is possible that the difficulty of voluntary dosing could be increased if the dose is associated with discomfort. However, the purpose of training and positive reinforcement is to desensitize animals to negative stimuli, so it is possible that VO dosing would be more successful than OG dosing of test articles that cause discomfort.[15]

A final limitation of the VO dosing method is the potential for the positive reinforcement reward to act as a confounding variable on interpretation of the study data. We chose a treat primarily based on known acceptance by minipigs. However, we also considered that grape juice has limited ingredients likely to act as variables, and that the volume was relatively limited. Aside from water, the primary component of grape juice is sugar, with vitamins, minerals, and polyphenols present at low levels.[16] Simple sugars were not identified as having significant potential for interactions with drugs in recent review articles on food–drug interactions.[17,18] However, caloric content in a liquid can slow gastric emptying time compared with a noncaloric liquid, which is a variable in drug pharmacokinetics.[19] Although it does not appear that the small volume of juice and limited calories used in this study had sufficient calories to affect the pharmacokinetics of metronidazole, it is possible that a different test article and a different dose volume could present different results. Polyphenols can have pharmacokinetic effects, particularly related to cytokine P450 enzymes.[20] However, an analysis of the amount of polyphenols

present in the volume of grape juice animals received with this method suggest that it is below the level at which significant food effects, beyond those already presented with the animals' standard diet.[21] If polyphenols are of particular concern for a study, a different reward may be employed. Pigs in particular are relatively indiscriminate with their food preferences, and many options are available.[22] It is also not known whether the amount of liquid associated with the juice reward, in addition to the liquid volume of the dose formulation, could affect drug pharmacokinetics. In dogs, liquid volumes of ≤100 mL did not stimulate gastric contractions or acid secretion, although volumes of 150 to 500 mL did.[23] The volume of liquid administered in this study is near that threshold of 150 mL for animals of similar weight, albeit a different species. The effect of the reward on gastric pH is another factor we did not evaluate in this study.[24] Grape juice is acidic and pH is known to affect drug absorption.[18,25] We did not expect this to be a significant variable based on the limited volume of the reward, but a different reward may be employed if pH is a significant, known variable at the expected level of dilution. In the absence of data demonstrating this variability, it should not be assumed to be a concern. Food effect on pharmacokinetics is not the rule, and many drug safety studies are performed successfully with the animal in the fed state with inclusion of natural ingredient enrichment items in the diet.

Given these potential variables, it may be necessary to perform basic feasibility studies with the VO dosing method prior to use in a drug development project. This may not be as challenging as it may seem. It is only necessary to determine whether there is an effect on the pharmacokinetics, not to determine the mechanism. Such may be achieved by adding an additional arm to an early pharmacokinetic study using VO dosing. The additional costs and effort to do so may be well justified if they allow use of a dosing method that is less stressful for animals and personnel, reduces staffing needs, and eliminates potential attrition due to dosing complications on a repeat dosing study. It also is consistent with the animal welfare goal of refinement of study procedures. If it is determined that VO dosing does have a confounding effect on the drug pharmacokinetics, then there are data to provide a scientific justification for not employing it as an alternative method.

In conclusion, this study demonstrates a method for successfully administering a single dose of an aversive tasting drug to pigs and achieving a comparable pharmacokinetic profile as with OG dosing. These methods may be adapted to future studies to determine whether this method has broader applications for repeat dosing studies and for different drugs. VO dosing offers the possibility for refining oral dosing in minipigs to reduce animal stress, reduce time and labor, and reduce the risk of complications associated with OG dosing.

Acknowledgments

We thank the Charles River Laboratories 3Rs Working Group and Dr. Joseph Hampel, Charles River Laboratories, for scientific review of the study design and manuscript, and Cierra Stone, Dana Schneider, and Heather Davis, all from Charles River Laboratories, for technical support and feedback.

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding

This work was internally funded.

Author Contributions

DM Cooper: study concept, design, study director, primary manuscript author, and overall manuscript input and approval; A Rainey: development and conduct of VO dosing procedure, study conduct, coauthor of methods, results, and discussion sections on training and dosing procedures, and overall manuscript input and approval; C Rosenfeld: study design input, coauthor of pharmacokinetic methods, results, and discussion sections, and overall manuscript input and approval; D Raich: performed pharmacokinetic analysis, coauthor of pharmacokinetic methods, results, and discussion sections, and overall manuscript input and approval; and H Miller: performed bioanalytical analysis, author of bioanalytical methods and results sections, and overall manuscript input and approval.

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