

Euthanasia Method for Mice in Rapid Time-Course Pulmonary Pharmacokinetic Studies

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To develop a means of euthanasia to support rapid time-course pharmacokinetic studies in mice, we compared retroorbital and intravenous lateral tail vein injection of ketamine–xylazine with regard to preparation time, utility, tissue distribution, and time to onset of euthanasia. Tissue distribution and time to onset of euthanasia did not differ between administration methods. However, retroorbital injection could be performed more rapidly than intravenous injection and was considered to be a technically simple and superior alternative for mouse euthanasia. Retroorbital ketamine–xylazine, CO₂ gas, and intraperitoneal pentobarbital then were compared as euthanasia agents in a rapid time-point pharmacokinetic study. Retroorbital ketamine–xylazine was the most efficient and consistent of the 3 methods, with an average time to death of approximately 5 s after injection. In addition, euthanasia by retroorbital ketamine–xylazine enabled accurate sample collection at closely spaced time points and satisfied established criteria for acceptable euthanasia technique.

Matching the attributes of the euthanasia method to different applications and study designs is an important consideration in selecting the euthanasia method for an *in vivo* study. Methods of euthanasia should adhere to the *AVMA Guidelines on Euthanasia*, *ACLAM Task Force Guidelines on Euthanasia*, and other references reiterating similar principles.^{1,3,7,11,16} According to these guidelines, an acceptable euthanasia method is characterized by: (1) rapid loss of consciousness; (2) reliability; (3) safety of personnel; (4) irreversibility; (5) compatibility with study requirements; (6) minimal negative emotional effect on observers and personnel; and (7) compatibility with subsequent evaluation, examination, or use of tissue sample.^{1,7} The purpose of the current set of studies was to compare commonly accepted means of euthanasia in mice with a novel method: retroorbital ketamine–xylazine euthanasia.

Ketamine–xylazine is a commonly used combination for anesthesia and euthanasia in mice.^{4,14,28} In our experience, ketamine–xylazine is most often given intraperitoneally as an anesthetic combination. When used for euthanasia purposes, typically an overdose of the anesthetic is administered intraperitoneally followed by a secondary means of euthanasia, such as exsanguination, thoracotomy, or cervical dislocation.

For intravenous drug administration in mice, the retroorbital injection method is a technically simple, easily learned, reproducible, and rapid procedure, particularly as compared with intravenous tail vein dosing. Retroorbital injection has been shown to be interchangeable with the intravenous tail vein injection technique when parenteral access is desired in the mouse.^{9,10,12,18,20,22,29} The variability, technical demand, and other negative aspects of intravenous tail vein dosing in mice make the retroorbital method desirable.^{9,10,12,18,20,22,29} To minimize any potential associated pain or distress, retroorbital injections typically are given to anesthetized mice.¹⁵ This practice is feasible when mice are intended to recover after the injection; however, use of the retroorbital technique for euthanasia has not been

documented. One goal of the current study was to demonstrate the adherence of retroorbital injection of ketamine–xylazine to the previously stated principles regarding euthanasia, with emphasis on the humaneness of the technique.

We developed the retroorbital ketamine–xylazine euthanasia technique to support rapid time-course mouse pulmonary pharmacokinetics studies in drug development. For these types of studies, which involve direct delivery of compounds to the lungs, intratracheal dosing is often the preferred method because of its reproducibility, reliability, and translatability to the clinic setting.^{19,21,23,24,26,27} One key factor that affects the efficacy and potency of these drug candidates is their residence time in the lungs. Pharmacokinetics studies focus on the distribution, clearance, and metabolism of chemical or drug entities that are introduced into the body. Pulmonary pharmacokinetics parameters are often assessed in serum, lung tissue, and bronchoalveolar lavage fluid. Because of rapid local clearance of the compounds, the quality of these data relies on the precision of sample collection, particularly from early time points that often are within minutes of each other.

When pulmonary pharmacokinetics studies are performed in mice, groups of animals are euthanized at specific time points after dosing to enable collection of tissue samples for concentration measurements over a time course. The method of euthanasia chosen for these studies must be nontraumatic and incorporate the attributes of rapid onset, ease of execution, reproducibility, and the ability to preserve tissues and samples. Currently, pulmonary pharmacokinetics studies use a variety of euthanasia techniques, including CO₂ exposure, intraperitoneal barbiturate overdose, cervical dislocation, and decapitation.^{13,19,23,26} These methods, when used in rapid time-point pharmacokinetics studies, have attributes that can confound the results.^{6,8,11,17} Cervical dislocation and decapitation result in rapid death but are traumatic in nature. These techniques damage the trachea and cervical region, making it difficult or impossible to lavage the lungs after the procedure. These methods also confound the results by causing hemorrhage into various tissues and contaminating the lung tissue. Other euthanasia methods, such as CO₂ exposure and intraperitoneal

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pentobarbital overdose are less traumatic, but the time to death is delayed and variable, thus preventing precise timing for tissue harvest.^{2,5,8,11,17} Therefore, the goals of this study were to examine the utility of a novel method of euthanasia, retroorbital administration of ketamine–xylazine for euthanasia of mice. We here demonstrate its positive effect on the quality of in vivo data, and show that retroorbital administration of ketamine–xylazine meets the criteria for acceptable euthanasia. Retroorbital administration of ketamine–xylazine for euthanasia of mice is ideal for pulmonary pharmacokinetics studies and for any study needing a rapid, nontraumatic means of euthanasia.

Materials and Methods

The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully AAALAC-accredited.

Male BALB/cAnNCrI mice (approximate weight, 25 g; Charles River Laboratories, Wilmington, MA) were housed and fed in an environment in accordance with established principles under the *Guide for the Care and Use of Laboratory Animals*.¹⁶ They were free of pathogens, parasites, and opportunists. The mice were housed 5 animals per cage in standard polycarbonate cages on corn cob bedding (Bed-o'-cobs, The Andersons, Maumee, OH) and maintained on a 12:12-h light:dark cycle. They were fed ad libitum (Lab Diet 5001, Purina Mills International, St Louis, MO) and had unrestricted access to water. The mice were allowed to acclimate in our facility for 4 d prior to the study.

Production of fluticasone nanosuspension formulation for pharmacokinetics study dosing. Fluticasone (Sequoia Research Products, Oxford, UK) is a well-known, FDA-approved compound that could be used in a standard pulmonary pharmacokinetics study. An unique nanosuspension created at Pfizer was used. To produce the nanosuspension formulation of fluticasone, a bench-scale wet milling (micronization) device was used. Fluticasone, an appropriate amount of multisized glass beads (0.5 to 0.75 mm), and Tween 80 [0.5% (w/w) in PBS (pH 7.4); used as a surfactant and wetting agent] were combined in a scintillation vial. The mixture was stirred at 1200 revolutions per minute for 24 h with occasional shaking. The stock formulation then was harvested, and potency was checked by HPLC–diode array detection, and its solid state was checked by powder X-ray diffraction. Thermal gravimetric analysis with simultaneous differential thermal analysis (TGA/SDTA851e, Mettler,) was performed. Particle size distribution was determined on a Beckman Coulter LS 230 particle size analyzer using the small volume accessory (Beckman Coulter, Miami, FL). A polarization intensity differential scattering obscuration water optical model was employed. Particle size distribution was computed by software using Mie scattering theory (Beckman Coulter, Fullerton, CA). Formulation concentration, homogeneity, chemical stability, and solid-state stability were performed according to the same procedures described.

Comparison of retroorbital and intravenous techniques for ketamine–xylazine euthanasia. Two groups of 7 naïve mice were euthanized with 100 μ L of a 10:1 mixture of ketamine (100 mg/mL; Ketaset, Fort Dodge, Fort Dodge, IA) and xylazine (100 mg/mL; Anased, Lloyd Laboratories, Shenandoah, IA) delivered either retroorbitally or by injection into the lateral tail vein.

The retroorbital injection was given after a scruff restraint of the mouse, followed by injection of ketamine–xylazine into the retroorbital sinus through a 26-gauge needle inserted into the lateral canthus of either orbit. For intravenous tail vein injection, mice were placed under a heat lamp to promote vascular dilation and facilitate dosing. The mice then were restrained to

allow access to the tail while keeping the body still. The time to death after treatment was recorded for each technique. The time spent under the heat lamp was not included in the total procedural time for intravenous injections. The average time to death was determined from the end of the injection to the point of clinical death (lack of respiration and heart beat). After clinical death, a terminal blood sample was collected by cardiocentesis. Brain, heart, and lung tissue were harvested for determination of ketamine–xylazine concentration.

Brain, lung, and heart tissue was weighed and homogenized individually in 2 mL 70% acetonitrile in 15-mL plastic centrifuge tubes (Corning, Corning, NY). Blood samples were placed into plasma separator tubes (Microcontainer Plasma Separator Tubes, Becton–Dickinson, Franklin Lakes, NJ) and centrifuged at $7826 \times g$ for 6 min. The tissue and plasma samples were frozen at -80°C until analysis.

Determination of tissue ketamine–xylazine concentration.

Tissue ketamine–xylazine quantification was performed by using HPLC and mass spectrometry (Pharmacokinetics Dynamics and Metabolism Department, Pfizer, St Louis, MO). The analysis system comprised a triple-quadrupole mass spectrometer (API4000, Applied Biosystems, Foster City, CA) with an atmospheric pressure electrospray ionization source (MDS SCIEX, Concord, Ontario, Canada) and 2 pumps with a controller (LC-10ADvp, Shimadzu, Columbia, MD). A 10- μ L sample of homogenized tissue or plasma was injected onto an Altima-C18 column (2.1 \times 50 mm; 3.0 μ m; Alltech, Deerfield, IL) and eluted by a mobile phase with initial conditions of 2% solvent B for 1 min followed by a gradient of 2% solvent B to 50% solvent B (100% in the case of xylazine) over 2 min (solvent A: 95% H₂O–5% acetonitrile with 0.1% formic acid; solvent B: 100% acetonitrile with 0.1% formic acid); 50% solvent B (100% in the case of xylazine) then was held for 1 min, followed by an immediate return to initial conditions and maintained for 1 min, with a flow rate of 0.4 mL/min. By using the positive-ion mode, protonated molecules were formed by using an ion-spray voltage of 5000 V (2500 V in the case of xylazine), declustering potential of 56 eV (61 eV in the case of xylazine), entrance potential of 10 eV, and source temperature of 300 $^\circ\text{C}$ for ketamine (250 $^\circ$ for xylazine). The product ion was generated at a collision energy of 29 eV (18eV for xylazine). For measuring ketamine, mass transition of m/z 238.1 (221 xylazine) to m/z 124.8 (90 xylazine) was used. The peak areas of all the analytes, standards blanks, and internal standard were quantified by using Analyst 1.4.1 (MDS SCIEX, Ontario, Canada).

Pulmonary pharmacokinetics study. For this portion of the study, 72 naïve male BALB/C mice were dosed intratracheally with a 0.8-mg/mL nanosuspension of fluticasone at 1 mg/kg. After the animals were sedated with inhaled isoflurane (Aer-rane, Baxter, Norfolk, UK), each was restrained through a scruff hold. The mouth was held open with a thin metal wire placed below the upper incisors. The tongue was retracted forward to expose the back of the oral cavity. The fluticasone then was pipetted into the opening of the larynx. After dosing, the animals were returned to their cages.

All dosed animals were divided randomly into 3 arms; 6 mice from each group were euthanized at each of 4 designated time points after dosing (5, 15, 30, and 60 min) by use of 1 of 3 euthanasia methods: (1) inhalation of 100% CO₂ in a nonpre-charged 4-L chamber at a moderate fill rate; (2) intraperitoneal pentobarbital (100 mg/kg; Sleepaway, Fort Dodge), and (3) retroorbital injection of ketamine–xylazine (100 μ L of a 10:1 ketamine–xylazine solution; Ketaset, Fort Dodge; Anased, Lloyd Laboratories). At the point of clinical death (lack of heart beat

and respiration), a terminal blood sample, bronchoalveolar lavage fluid, and lung tissue was harvested for pharmacokinetics analysis.

Lung tissue was weighed and homogenized in 3 mL 70% acetonitrile in 15-mL plastic centrifuge tubes (Corning). Blood samples were placed into plasma separator tubes (BD Micro container Plasma Separator Tubes, Becton–Dickinson) and centrifuged at $7826 \times g$ for 6 min. The tissue and plasma samples were frozen at -80°C until analysis.

Determination of tissue concentration of fluticasone. Analysis of plasma and lung samples for fluticasone concentration was performed by using HPLC–mass spectroscopy according to the same methods described for ketamine, with the following exceptions: the initial 10- μL injected sample was eluted by a mobile phase with initial conditions of 10% solvent B for 1 min, followed by a gradient of 10% solvent B to 100% solvent B over 2 min (solvent A: 95% H_2O –5% acetonitrile with 0.1% formic acid; solvent B: 100% acetonitrile with 0.1% formic acid), 100% solvent B held for 1 min, followed by an immediate return to initial conditions maintained for 1 min, with a flow rate of 0.4 mL/min. The declustering potential was 80eV with a source temperature of 400°C . The product ion was generated at collision energy of 19 eV for measuring the fluticasone mass transition of m/z 502.1 to m/z 313.7.

The half-lives of fluticasone in the lungs and plasma of the study mice were calculated based on the theoretical concentration of fluticasone after intratracheal dosing (for lung tissue) or first measurement (in the case of plasma). The starting plasma concentration is different for each euthanasia technique due to the different time to onset of death for each method.

Statistical methods. A 2-tailed t test (LabStats, Computer Lab Solutions, Farmington, UT) was used to compare the means of the tissue concentrations of the retroorbital versus the lateral tail vein injection. A confidence interval of 95% or a P value of less than or equal to 0.05 was considered statistically significant.

In addition, 1-way ANOVA (LabStats, Computer Lab Solutions) was used to compare the means of lung fluticasone concentration at each time point in the pharmacokinetics study among the 3 euthanasia techniques and to compare the means of plasma fluticasone concentration at each time point in the pharmacokinetics study among the 3 methods. A confidence interval of 95% or a P value of less than or equal to 0.05 was considered statistically significant.

Results

Both retroorbital and intravenous administration of ketamine–xylazine to mice rapidly resulted in clinical death (lack of heart beat and respiration). Average time to clinical death for the retroorbital method was 5 s, and the average time to death for the intravenous method was 3 s (the time from end of injection to clinical death). The primary difference between the 2 techniques was the preparation time required for the injection. The retroorbital method requires approximately 10 s to execute. With intravenous tail vein injection, the tail must be prewarmed to dilate the vessel, with the mouse restrained in a way that allowed access to the tail for the injection. The total time from cage removal to death by tail vein injection was approximately 60 s, not including the time spent under the heat lamp for vessel dilation. Therefore the difference in time between the 2 methods was approximately 50 s for each animal, with the retroorbital method less time-consuming overall.

The average concentrations of ketamine and xylazine in the brain, lung, heart, and plasma after retroorbital euthanasia and intravenous euthanasia (Table 1) were compared with 2-tailed t

tests. The tests revealed no significant differences in mean tissue concentrations between the 2 methods.

In the fluticasone pharmacokinetics study, the average time to clinical death for CO_2 inhalation was 262 s, for intraperitoneal pentobarbital was 253 s, and for retroorbital ketamine–xylazine was 5.3 s (Figure 1). Statistical testing was not done to compare average times to clinical death in light of the pronounced differences in time between retroorbital ketamine–xylazine and the other 2 methods.

The concentration of fluticasone in plasma and homogenated lung tissue was determined at various time points (Figures 2 and 3). At 5 min after dosing, the lung concentration of fluticasone was different in the 3 groups euthanized with different techniques. The fluticasone level was the highest in the ketamine–xylazine euthanized group. The corresponding plasma level is the lowest when compared with other methods at this time point. Plasma fluticasone levels were higher in the CO_2 euthanasia group as compared with the other 2 methods of euthanasia. By 30 min, the plasma and lung levels were almost identical from all 3 groups of animals. The variability in the data for each group decreased at later time points as the clearance rate is slower at these time points.

For all 3 euthanasia methods, mean lung concentrations of fluticasone were significantly different ($P < 0.05$) by 1-way ANOVA at the 5- and 15-min time points; mean plasma fluticasone at these time points were different between these 3 methods as well. At the 30-min time point, drug concentrations in lung did not differ significantly between the euthanasia methods, but differences in plasma concentration between methods remained at the 30-min point. All significant differences in plasma and lung concentrations between the 3 different methods were lost at 60 min.

Table 2 shows the calculated half life of fluticasone in lung and plasma. The pulmonary half life was calculated based on the theoretical starting concentration of fluticasone. The plasma half life was calculated based on the first concentration since the plasma concentration is 0 at time 0. Depending on the euthanasia technique, the half life of fluticasone in lung and plasma varied by as much as 5 min.

Discussion

The combination of ketamine and xylazine is a common anesthetic regimen in mice.^{4,14,28} The use of this combination by retroorbital injection for euthanasia has not been reported previously. Comparison of retroorbital and intravenous injection of ketamine–xylazine revealed that the concentrations of ketamine–xylazine do not differ between these methods. This finding suggests that the target organs and mechanism of action for both methods are likely identical. This information, combined with the time required to complete each procedure, suggests that the retroorbital method is a superior option when a fast, technically simple, and nontraumatic means of euthanasia is desired.^{9,10,12,18,20,22,29}

An important factor demonstrated in these studies was that retroorbital injection of ketamine–xylazine met all established guidelines for euthanasia. This method was rapid, easily reproducible, irreversible, required minimal restraint, and could be learned quickly. It is also safe for personnel, is humane and nontraumatic, and can accommodate study requirements. These attributes are in line with published accepted principles of euthanasia.^{1,7,11,16,3} Several sources, including the 2nd edition of *The Mouse in Biomedical Research* advocate the use of anesthesia during retroorbital injections.¹⁵ This guideline is due, in part, to the theorized pain and distress involved with

retroorbital injections in recovering mice. The reference does not mention use of retroorbital injections for euthanasia purposes. As we present here, clinical death after retroorbital injection of ketamine–xylazine was rapid—within seconds of injection—thus minimizing concerns of the technique causing anything more than momentary pain or distress. As demonstrated in the fluticasone pharmacokinetics study, this method allowed collection of tissue samples at precise time points.

Cervical dislocation and decapitation are both rapid means of euthanasia, but they can cause complications that are not amenable to certain studies. Some of these complications include hemorrhage into the thorax or lungs, bruising and damage to cervical structures and trachea, and even adverse changes to abdominal organs.^{8,11,17} Commonly used nontraumatic means of euthanasia, such as CO₂ or barbiturate overdose, resulted in variable time to death. Slow onset of death does not support sample collection at precise time points. In contrast, retroorbital injection of ketamine–xylazine enabled us to adhere to tight, rapid time lines and allowed parallel processing of several animals. The slow and variable onset of death after CO₂ exposure and pentobarbital overdose hindered precision in sample collection and directly affected the pharmacokinetics profile, especially at early time points. Other concerns with the use of CO₂ in pharmacokinetics studies involve its potential negative effect on the metabolism of compounds due to pH changes as well as potential adverse effects to various tissues.^{2,6}

Because the clearance of drug candidates from the lungs is most rapid upon delivery, these rapid changes in lung concentration must be captured during the early time points for an accurate calculation of compound half life. Fluticasone was present and measurable in plasma as early as 5 min after administration. The lung levels of fluticasone were the highest in the retroorbitally euthanized animals at the early time points and second highest in plasma at the same time point (because of more drug remaining in lung tissue). Because of the rapidity of the retroorbital euthanasia method, more compound was captured in the lungs, before clearance into plasma. This finding demonstrates the necessity of a rapid onset and efficient euthanasia method to capture these dynamic events. The data also clearly indicate that the method of euthanasia influenced the perceived pharmacokinetics profile.

The lung and plasma concentrations of fluticasone differed significantly between the 3 euthanasia methods at the 5-min time point. At the 15-min time point, this variability was less prominent but still significant; these differences became insignificant at later time points. This finding demonstrated that the method of euthanasia can directly affect the data at the closely spaced early time points, where precision in sampling time is most crucial. The variability between methods could be due to additional factors, such as euthanasia metabolism effects, prolonged and variable time to death, and procedure-related stress. These factors should be addressed in future studies.

For the pentobarbital-injected mice, the plasma level of fluticasone at 5 min was low in comparison with the other methods, but the lung levels of fluticasone were relatively high. One potential reason for these findings is that pentobarbital may affect the metabolism of fluticasone and its clearance from the lungs into the circulation, thus decreasing systemic availability. This theory is supported in part by a study showing similar phenomena with oxacillin levels in rats.²⁵ In that study, oxacillin was administered to rats that were either anesthetized with pentobarbital or remained conscious. Samples of portal and arterial blood were obtained to measure plasma concentrations of oxacillin in both groups. The metabolism of oxacillin

Table 1. Concentrations of ketamine and xylazine in various tissues after clinical death

Tissue	Route of administration	Average concentration (µg/mL)	
		Ketamine	Xylazine
Brain	Retroorbital	85.67	5.69
	Intravenous	88.49	5.92
Lung	Retroorbital	653.71	56.71
	Intravenous	521.53	47.33
Heart	Retroorbital	335.57	15.83
	Intravenous	374.29	16.42
Plasma	Retroorbital	5091.43	543.57
	Intravenous	4759.57	390.35

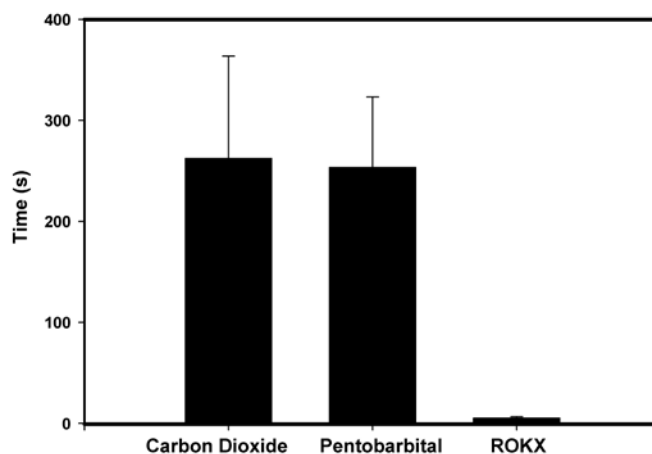


Figure 1. Time to clinical death. ROKX, retroorbital injection of ketamine–xylazine.

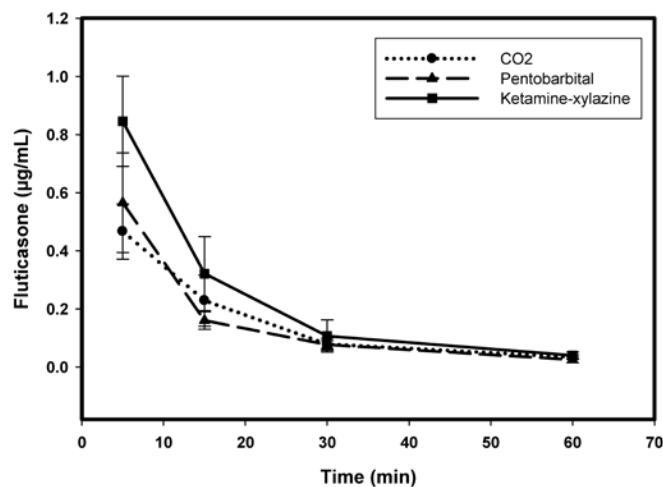


Figure 2. Pharmacokinetics profile of fluticasone in the lungs as determined by using the 3 different euthanasia techniques.

was significantly different in the 2 groups. Further, the hepatic clearance of oxacillin was lower and mean absorption time and absolute bioavailability both higher in anesthetized rats. Pentobarbital could have a similar effect on the drug clearance from lung in our study.

The calculated pulmonary half life of fluticasone varied with the euthanasia technique, most likely due to a direct effect of the method of euthanasia. The calculation of half-life requires accurate measurement of the starting concentration in the tissue. For a euthanasia method with a long onset time, accurately

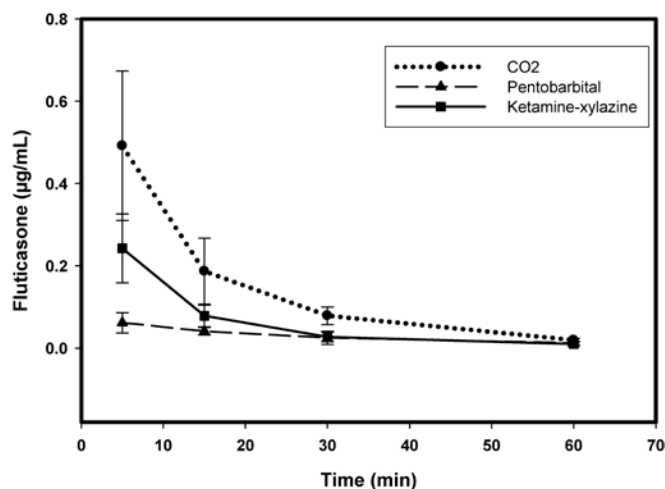


Figure 3. Plasma pharmacokinetics profile of fluticasone as determined by using the 3 different euthanasia techniques.

Table 2. Half-life of intratracheal fluticasone in plasma and lung

	Euthanasia method	Calculated half-life (min)
Lung	Carbon dioxide	8
	Pentobarbital	9
	Ketamine-xylazine	13
Plasma	Carbon dioxide	12
	Pentobarbital	17
	Ketamine-xylazine	13

capturing these time sensitive tissue concentrations would be impossible.

The retroorbital ketamine-xylazine injection technique offers an option of mouse euthanasia that might be used in various areas of research where rapid, nontraumatic, technically simple, and consistent means of euthanasia are desired. This technique satisfies the requirements for sound euthanasia as described in guidelines from the AVMA, ACLAM Task Force, and others. Future studies should explore the utility of euthanasia through retroorbital ketamine-xylazine in various study designs and the possible application of this technique in other rodent species.

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