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A Pharmacokinetic-Pharmacodynamic Study of Intravenous Midazolam and Flumazenil in Adult New Zealand White—Californian Rabbits (*Oryctolagus cuniculus*)

Frédérik Rousseau-Blass,[1,2] Alastair E Cribb,[3] Francis Beaudry,[1,2] and Daniel SJ Pang[1,2,4,*]

Flumazenil, a competitive $GABA_A$ receptor antagonist, is commonly used in rabbits to shorten sedation or postanesthetic recovery after benzodiazepine administration. However, no combined pharmacokinetic (PK) and pharmacodynamic (PD) data are available to guide its administration in this species. In a prospective, randomized, blinded, crossover study design, the efficacy of IV flumazenil (FLU; 0.05 mg/kg) or saline control (SAL; equal volume) to reverse the loss of righting reflex (LORR) induced by IV midazolam (1.2 mg/kg) was investigated in 15 New Zealand white rabbits (2.73 to 4.65 kg, 1 y old). Rabbits were instrumented with arterial (central auricular artery) and venous (marginal auricular vein) catheters. After baseline blood sampling, IV midazolam was injected (T0). Flumazenil or saline (FLU/SAL) was injected 30 s after LORR. Arterial blood samples were collected at 1 and 3 min after midazolam injection, and at 1, 3, 6, 10, 15, 21, 28, 36, 45 and 60 min after injection with flumazenil. Plasma samples for midazolam, 1-OH-midazolam and flumazenil were analyzed using high performance liquid chromatography-high-resolution mass spectrometry and the time to return of righting reflex (ReRR) was compared between groups (Wilcoxon test). FLU terminal half-life, plasma clearance and volume of distribution were 26.3 min [95%CI: 23.3 to 29.3], 18.74 mL/min/kg [16.47 to 21.00] and 0.63 L/kg [0.55 to 0.71], respectively. ReRR was 25 times faster in rabbits treated with FLU (23 [8 to 44] s) compared with SAL (576 [130 to 1141] s; 95%CI [425 to 914 s]). Return of sedation (lateral recumbency) occurred in both groups (7/13 in FLU; 12/13 in SAL) with return of LORR in a few animals (4/13 in FLU; 7/13 in SAL) at 1540 [858 to 2328] s. In the population and anesthesia protocol studied, flumazenil quickly and reliably reversed sedation induced by midazolam injection. However, the potential return of sedation after flumazenil administration warrants careful monitoring in the recovery period.

Abbreviations: BZD, Benodiazepines

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Benzodiazepines (BZD) are a class of sedatives-hypnotics commonly used in exotic companion and laboratory animals (birds, reptiles, and small mammals).[10,18] Their mechanism of action is mediated by enhancing γ-aminobutyric acid (GABA) affinity for the $GABA_A$ receptor, and results in sedation, anxiolysis, and muscle relaxation with minimal cardiorespiratory depression.[17] The short-acting BZD, midazolam, is frequently used in rabbits as a sole agent, or in a premedication combination.[11,23,24] The ability to deliver midazolam by the intramuscular route (in contrast to diazepam) is useful in rabbits, as their temperament often means that sedation is required for noninvasive procedures such as radiography or intravenous catheterization.[11]

The increasing popularity of rabbits, both as pets and in research, has created a greater demand for rabbit anesthesia.[14] Unfortunately, the risk of perianesthetic mortality remains higher in rabbits as compared with dogs or cats, with the majority of deaths occurring during the recovery period.[12,34] This tendency toward death during recovery is likely multifactorial, with possible causative factors including the continued depressive effects of sedative and anesthetic drugs during recovery, a period when physiologic monitoring and surveillance is frequently decreased.[12] Therefore, shortening the recovery period through pharmacological antagonism of drugs is an attractive approach that has already shown benefits in other animals and in humans.[26,30]

Flumazenil (FLU) is a selective $GABA_A$ receptor antagonist that antagonizes the clinical effects of midazolam through competitive inhibition at the benzodiazepine allosteric site of the $GABA_A$ receptor.[31] Its use reduces both recovery and discharge times after benzodiazepine sedation in humans.[39] However, the potential for return of sedation after FLU antagonism has been reported.[7,21,39] The available literature on the use of FLU in rabbits is very limited, with the only data found in reports that used considerable variations in dose (0.02 to 0.1 mg/kg IV, IM or SC).[5,16,44] Furthermore, the quality and duration of recovery and the potential for resedation, is rarely described or quantified, making the frequent use of FLU in clinics largely anecdotal rather than evidence-based.

The objectives of this study were to investigate the pharmacokinetic (PK) and pharmacodynamic (PD) parameters of FLU

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[1]Faculty of Veterinary Medicine, Université de Montréal, Saint-Hyacinthe, Québec, Canada; [2]Groupe de recherche en pharmacologie animale du Québec (GREPAQ), Université de Montréal, Saint-Hyacinthe, Québec, Canada; [3]Cummings School of Veterinary Medicine, Tufts University, N Grafton, Massachusetts; [4]Department of Veterinary Clinical and Diagnostic Sciences, Faculty of Veterinary Medicine (UCVM), University of Calgary, Calgary, Alberta, Canada
[*]Corresponding author: Email: dsjpang@ucalgary.ca

and midazolam in rabbits, including the risk of resedation after FLU administration. A secondary objective was to assess the degree of sedation using a modified sedation scale in rabbits. Our hypotheses were that: 1) PK of FLU in rabbits will allow a dose of 0.05 mg/kg to fully and safely reverse the sedative effects of midazolam; and 2) The modified sedation scale will allow differentiation of sedated and unsedated rabbits.

Materials and Methods

Animals. Fifteen male ($n = 7$) and female ($n = 8$) New Zealand white-Californian cross rabbits (range 2.73 to 4.65 kg, 1 y old), purchased from a commercial vendor (Ferme Laobec, Acton Vale, Québec, CA) were used. The rabbits were free from *Pasteurella multocida*, *Salmonella spp.*, *Bordetella bronchiseptica*, *Treponema cuniculi*, *Clostridium perfringens*, *Mycobacterium spp.*, rotavirus, poxvirus, calicivirus, endoparasites and ectoparasites (PCR and fecal flotation tests). They were fed a commercial pelleted diet (5079-U.S. Charles River Autoclavable Rabbit food, Charles River Laboratories, QC, Canada) and autoclaved hay from a local supplier (Ferme Lumunick, QC, Canada) with tap water provided ad libitum. Rabbits were housed in individual cages (elevated cages, with perforated plastic flooring, without bedding; 70 cm × 70 cm × 45 cm, Allentown, NJ) with enrichment (Jingle Ball and Dumbbells, Bio-Serv, NJ) in an environmentally controlled housing room (humidity 35 [25 to 55] %, temperature 18 [17 to 19] °C) and a light-dark cycle of 12:12 h, with lights on at 0600 h (270 [245 to 320] lux, median [range]). Each animal had weekly access to a pen (4 × 4 meters) filled with softwood pine shavings and enrichment toys. After an acclimation period of 2 wk, rabbits were enrolled in the study. All rabbits were assessed twice daily, including observation of general appearance, activity level, feces production and an evaluation of appetite and water consumption.

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Université de Montréal (18-Rech-1973). All procedures were conducted in accordance with the principles outlined in the current Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care).[14] All procedures were performed during the light phase (0800 to 1600) and all experiments were performed over 2 periods of 5 d, separated by 14 d.

Study design. This was a prospective, randomized, blinded, crossover trial with 2 treatment arms separated by a 2 wk washout period. During the first arm of the study, rabbits were randomly allocated (random number generator, www.random.org) to receive a standard dose of midazolam (Sandoz, QC, Canada), followed by either flumazenil (FLU; Sandoz, QC, Canada) or saline control (SAL). Each rabbit received the opposite treatment in the second arm. Initial treatment order was randomized (same method) and investigators were blind to treatment allocation. Each rabbit was weighed (SRV930 scale, SR Instruments, NY) and transported to a separate room for the experimental procedure. Rabbits were gently restrained with an adjustable fabric body wrap (Bunny Snuggle, Lomir Biomedical, QC, Canada). Rabbits were habituated to handling and restraint during the 2 wk holding period. The fur on the exterior surface of both ears was manually depilated and a local anesthetic cream (Lidocaine 2.5%, Prilocaine 2.5%, EMLA, AstraZeneca, ON, Canada) applied with an occlusive dressing over the marginal auricular vein and central auricular artery (cream applied over approximately 2 cm^2). The dressing was left in place for a minimum of 30 min, after which the skin was aseptically prepared (0.5% chlorhexidine and alcohol swabs) for cannulation of the artery and vein using over-the-needle catheters (24-gauge, Becton, Dickinson and Company, NJ). An arterial blood pressure transducer (TruWave, Edwards LifeScience, CA) was positioned and zeroed at the level of the heart (sternum) and connected to the arterial catheter using noncompliant tubing (Pressure tubing, Edwards LifeScience, CA) filled with heparinized saline. Arterial blood pressures were displayed on a multiparametric monitor (LifeWindow LW6000, Digicare Biomedical, FL). Once all catheters and instrumentation were installed, the baseline for systolic, mean and diastolic arterial blood pressures were recorded, followed by baseline arterial blood sampling. Subsequently, all rabbits received a midazolam bolus (1.2 mg/kg IV). The bolus was hand-injected over 5 s. Completion of the injection was the baseline time (T0) to which all other times were referenced. Based on treatment allocation, FLU (0.05 mg/kg IV) or an equal volume of SAL was injected 30 s after loss of righting reflex (hand-injected over 5 s). Arterial blood samples were collected 1 and 3 min after midazolam injection and at 1, 3, 6, 10, 15, 21, 28, 36, 45, and 60 min after treatment (FLU/SAL) injection (Figure 1).

Behavioral assessment. Time from midazolam injection (T0) to head down, lateral recumbency, and loss of righting reflex (LORR) were recorded. Lateral recumbency and righting reflex were tested by attempting to place each rabbit in lateral or dorsal recumbency, respectively. Handling was standardized and performed by the same 2 investigators throughout the study. Lateral recumbency was tested at 2.5 min after midazolam injection and then every 2 min. The righting reflex was tested 5 min after midazolam injection and every 2 min subsequently. Once lateral recumbency and LORR was achieved, the animal was not retested until return of the righting reflex (ReRR). The ReRR occurred spontaneously, without stimulation. For both lateral recumbency and righting reflex, a maximum of 3 trials were attempted. The delay between treatment injection (FLU/SAL) and ReRR was recorded. The delay between ReRR and any return to lateral recumbency or a second LORR was also recorded. Placement in lateral recumbency was tested every 2 min (maximum of 3 trials) after ReRR. LORR was tested every 2 min (maximum of 3 trials) after a return to lateral recumbency. Sedation levels were assessed using a modified version of the sedation scale described previously (Figure 2).[51] Sedation was assessed 4, 10, and 30 min after midazolam injection. If a rabbit displayed LORR, sedation was assigned the maximal score (14/14). Assessment of body position was performed 30 min after return to the cage. Exclusion criteria included any deviation from study protocol due to technical difficulty (failure to place venous or arterial catheter) or delayed/absent LORR.

Blood sampling. Arterial blood sampling technique was as follows: a 3mL syringe (Luer lock, Terumo, NJ) was connected to a 3-way stop cock (Smiths Medical ASD, OH) placed between the catheter extension and noncompliant tubing (Pressure tubing, Edwards LifeScience, CA). Three mL of blood was initially withdrawn (twice the line volume) followed by approximately 1 mL into a heparinized syringe (AirLife, CareFusion, France). Air was expelled and the sample immediately placed on dry ice for a maximum of 15 min before centrifugation (sample did not freeze). The initial 3mL of blood was reinjected into the arterial catheter and the line flushed with 1 mL of heparinized saline. Samples were centrifuged at 2,000 *g* for 10 min within 10 min of collection. A volume of 300 µL of plasma was withdrawn from the sample and stored on dry ice until the end of the experimental period (approximately 6 h), then stored at -80 °C until analysis.

Plasma sample analysis. Drugs were extracted from rabbit plasma using a simple protein precipitation method. A total of

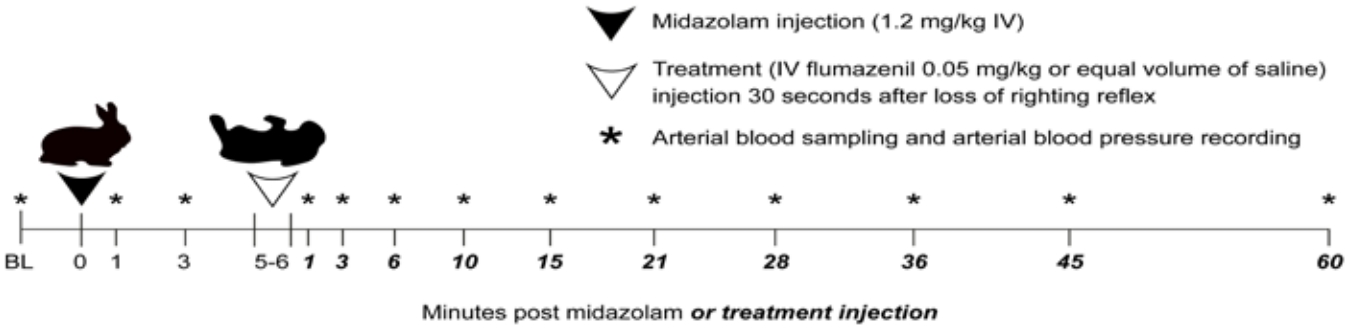

Figure 1. Experimental timeline. Randomized, controlled, cross-over study design with each rabbit receiving flumazenil or saline, separated by 2 wk between treatments. Arterial blood samples collected and analyzed immediately after collection. n = 15 in each treatment group.

50 µL of sample was mixed with 250 µL of internal standard solution (500 ng/mL of d_5-FLU, d_6-midazolam and d_5-1-OH-midazolam in 50:50 methanol) in a 1.5 mL centrifuge tube. The sample was then vortexed vigorously and allowed to rest for 10 min at room temperature prior to centrifugation. Samples were centrifuged at approximately 12,000 *g* for 10 min and 200 µL of the supernatant was transferred into an injection vial for analysis. The HPLC system was a Thermo Scientific Vanquish FLEX UHPLC system (San Jose, CA). Chromatography was achieved using a gradient mobile phase along with a microbore column Thermo Biobasic Phenyl 50 × 1 mm, with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile (A) and water (B) (both fortified with 0.1% of formic acid) at a ratio of 5(A):95(B). From 0 to 1 min, the ratio was maintained at 5(A):95(B). From 1 to 3 min, the mobile phase ratio was set to 85(A):15(B). The mobile phase composition ratio was reverted to the initial conditions and the column was allowed to reequilibrate for 5 min for a total run time of 8 min. The flow rate was fixed at 100 µL/min and 2 µL of samples were injected. A Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer (San Jose, CA) was interfaced with the UHPLC system using a pneumatic assisted heated electrospray ion source. Mass spectrometry detection was performed in positive ion mode and operating in scan mode at high-resolution, and accurate-mass (HRAM). Nitrogen was used for sheath and auxiliary gases which were set at 10 and 5 arbitrary units. The heated ESI probe was set to 4000 V, auxiliary gas set to 200 °C and the ion transfer tube temperature was set to 300 °C. The scan range was set to m/z 200 to 600. Data was acquired at a resolving power of 140,000 (FWHM) using automatic gain control target of 3.0×10^6 and maximum ion injection time of 200 msec. Targeted drug quantification was performed at MS^1 level using specific precursor masses based on the monoisotopic masses (that is $[M+H]^+$ ions). Quantification was performed by extracting specific precursor ions using a 5 ppm mass window and peak area ratio with specific stable isotope-labeled internal standard. Instrument calibration was performed prior to all analyses and mass accuracy was below 1 ppm using Thermo Pierce calibration solution and automated instrument protocol. The precision and accuracy of the method has met generally accepted performance criteria in bioanalytical chemistry.[15] The limit of quantification was 5 ng/mL for each analyte.

Posture	Normal	**0**
	Sedated but standing/sitting with head up	**1**
	Lying sternally, head up	**2**
	Lying sternally, head down	**3**
	Lying laterally, head up	**4**
	Lying laterally, head down	**5**
Palpebral reflex	Brisk	**0**
	Slow, but eyelids fully close	**1**
	Slow, with partial closure of eyelids	**2**
	Absent	**3**
Eye position	Central	**0**
	Rotated forwards/downwards but not obscured by nictitating membrane	**1**
	Rotated forwards/downwards and obscured by nictitating membrane	**2**
	Comment if nystagmus	**-**
Orbital tightening	Totally open/round	**0**
	Partially close (<50% of eye closure)	**1**
	Closed (>50% of eye closure)	**2**
General appearance/attitude	Awake and normal	**0**
	Tranquil	**1**
	Stuporous	**2**

Figure 2. Sedation levels assessed using a modified version of the sedation scale.[51]

Pharmacokinetic and pharmacodynamic modeling. Pharmacokinetic parameters for midazolam and FLU were determined by noncompartmental analysis using PKSolver, a freely available add-in program for Microsoft Excel (Microsoft, Redmond, WA).[53] All pharmacokinetic analyses were carried out by designating the time of administration as t = 0. All calculations were based on the time at which samples were actually taken. To assess the drug concentration at which specific behavioral events occurred, the plasma concentration at the time the event occurred was extrapolated from the concentrations measured at the 2 time points bracketing the event. The following formula was used, where C_A = the concentration at the time point prior to the event (T_A; minutes) and C_B = the concentration at the time point (T_B; minutes) after the event. C_E and T_E (seconds) represent the drug concentration and the time of the event (for example, loss of righting reflex, return of righting reflex), respectively.

$$C_E = \left(C_A\right) - \left(\left(C_A - C_B\right) \div \left(T_B - T_A\right) \times \left(T_E - \left(T_A \times 60\right)\right) \div 60\right)$$

Statistical analyses. The distribution of data was assessed with a D'Agostino-Pearson omnibus normality test. If data did not approximate a normal distribution, nonparametric tests were applied. The delay for ReRR, return to lateral recumbency and return to 2nd LORR were compared between both groups with a Wilcoxon test. The plasma concentrations of midazolam and FLU at these behavioral outcomes were compared with a 2-tailed paired *t* test. Time to head down, lateral recumbency and LORR represent the mean value for each rabbit from the 2 trials. No treatment differences were detected before the FLU treatment injection (data not shown). To assess the incidence of 2nd LORR, body position (LORR or not) was evaluated with a Fisher's Exact test (one-tail) from ReRR until 60 min after the treatment injection. A one-tail test was used based on prediction of the direction of the association before collecting data (FLU reducing signs of sedation). Return to lateral recumbency after ReRR was analyzed using the same method. Dose-response curves were generated based on the cumulative number of animals showing response (for example LORR) and plasma log concentrations. The half-maximal effective concentration (EC50) was determined using the standard log(agonist) compared with response function to generate a nonlinear response curve (GraphPad Prism 6.07, GraphPad Software, CA). Spearman rank correlation coefficient was used to assess the correlation between initial midazolam concentration and time to LORR or plasma concentration at LORR. Mean arterial blood pressures (MAP) were compared between groups and timepoints with a mixed linear model using treatment, time and interaction between these 2 parameters as fixed effects. The significance level (α) was set at 5%. Parametric data are presented as mean ± SD and nonparametric data are presented as median [range]. All data are presented with 95% confidence interval (95%CI) of the difference. Statistical analysis was performed with commercial software (GraphPad Prism 6.07, GraphPad Software, CA). An a priori power analysis estimated 12 rabbits (6 per group) were required to detect a mean difference of 30 s (standard deviation: 15 s) between FLU and SAL for the return of righting reflex (based on pilot data) with a power of 0.9 (1 - β error), and α of 5% (G*Power, Heinrich-Heine-Universität Düsseldorf, Germany). The data supporting the study results are available in an electronic repository: https://doi.org/10.7910/DVN/2ODA3H.

Results

All rabbits (n = 15) completed the study. Two female rabbits were excluded from all data analysis, as one never achieved LORR after midazolam injection and one had a longer ReRR with FLU than SAL (>100 standard deviations from treatment group mean). Plasma analysis confirmed that both rabbits had received the intended drugs and PK data showed that plasma profiles were similar to the rest of the sample population (data not shown). Therefore, data were analyzed from 7 males and 6 females.

The pharmacokinetic parameters of midazolam, 1-OH midazolam and FLU are presented in Tables 1, 2 and 3, respectively. Figures 3, 4 and 5 show the plasma concentration curves over time from a representative rabbit for midazolam, 1-OH-midazolam and FLU, respectively. No correlation was detected between the initial concentration of midazolam and the time to LORR (P = 0.29, Spearman r = -0.32 95%CI [-0.74 to 0.31]) or between the initial midazolam concentration and the plasma concentration of midazolam when LORR occurred (P = 0.99, Spearman r = 0.003 95%CI [-0.56 to 0.57]). FLU administration did not influence midazolam and 1-OH-midazolam pharmacokinetics (Tables 1 and 2).

After the midazolam injection, times to achieve head down position and lateral recumbency were 69 [36 to 108] s and 164 [157 to 192] s, respectively. The time from midazolam injection to LORR was 326 [307 to 521] s, which occurred at a plasma midazolam concentration of 802 [633 to 1083] ng/mL. The midazolam EC_{50} for LORR was 810 ng/mL (95%CI [789 to 831] ng/mL).

The ReRR was 25 times faster in the FLU group (23 [8 to 44] s) than in the SAL group (576 [130 to 1141] s; P < 0.001, 95%CI [425 to 914 s], Figure 6) and occurred at a significantly higher midazolam concentration [(821 [497 to 1148] as compared with 456 [333 to 744] ng/mL; P < 0.0001)]. The FLU EC_{50} for ReRR was 165 ng/mL (95%CI [122 to 224] ng/mL). Rabbits in the FLU group remained sternal significantly longer after ReRR (554 [354 to 1646] than did the SAL group (150 [91 to 1359] s; P = 0.016 95%CI [204 to 1210]), and their return to lateral recumbency also occurred at a lower midazolam concentration (54 [40 to 68] compared with 442 [334 to 556] ng/mL; P = 0.03). Fewer rabbits returned to lateral recumbency after ReRR in the FLU group (54%; 7/13) as compared with SAL group (92%; 12/13) (P = 0.04). Return to lateral recumbency occurred when FLU plasma concentration fell below a median concentration of 57.4 ng/mL [40.1 to 69.3], which took place at 1186 [726 to 2275] s. A number of rabbits reached LORR a second time after ReRR, with similar incidence between FLU (31%; 4/13) and SAL (54%; 7/13) groups (P = 0.21). The timing of the 2nd LORR was earlier for FLU (972 [858 to 1480] s) than for SAL (1840 [1147 to 2328] s; P = 0.012, 95%CI [79 to 1266 s]).

Sedation scores were not different between treatment groups after midazolam and before treatment injections (p > 0.99). FLU treatment significantly decreased sedation scores at the first (p < 0.001, 95%CI [3.1 to 10.5]) but not the second (P = 0.274, 95%CI [-1.2 to 6.2]) timepoint after injection (Figure 6 B).

No differences between groups were detected in mean arterial blood pressure at any time point (P = 0.75). Mean arterial blood pressure was lower than baseline from 15 min after treatment injection in FLU (P < 0.001, Figure 7) and SAL groups (P = 0.035). After midazolam injection, 18/26 (73%) rabbits displayed nystagmus of 2 to 5 min duration and one rabbit exhibited teeth grinding in both treatment arms. After FLU injection, no rabbits displayed agitation or other adverse behavioral effects. Thirty minutes after returning to their cage, no rabbits could be placed in lateral or dorsal recumbency.

Table 1. Pharmacokinetic parameters of midazolam alone (1.2 mg/kg IV) or in the presence of flumazenil (0.05 mg/kg IV) in 13 adult New-Zealand white-Californian cross rabbits. $t_{1/2}$: terminal elimination half-life; T_{max}: time at maximal plasma concentration; C_0: extrapolated plasma concentration at time 0; AUC_{0-t}: area under the curve of plasma concentration over time from time 0 to final timepoint; $AUC_{0-\infty}$: area under the curve of plasma concentration over time from time 0 to infinity; MRT: mean residence time; Cl: clearance; V_{ss}: volume of distribution at steady state.

		Midazolam alone (n = 13)		Midazolam with flumazenil (n = 13)		Paired t test
Parameters	Units	Mean value	95% CI	Mean value	95%CI	P value
$t_{1/2}$	min	29.1	26.0–32.3	30.6	26.2–35.0	0.55
C_0	ng/mL	2933	2507–3359	2920	2323–3517	0.89
AUC_{0-t}	ng·min/mL	28579	26752–30406	28999	25967–32030	0.80
$AUC_{0-\infty}$	ng·min/mL	34943	32727–37118	35264	31139–39388	0.88
MRT	min	36.71	33.22–40.20	35.72	31.05–40.39	0.71
Cl	mL/min/kg	34.67	32.57–36.77	35.45	30.50–40.40	0.75
V_{ss}	L/kg	1.27	1.15–1.39	1.24	1.09–1.39	0.73

Table 2. Pharmacokinetic parameters of 1-OH-midazolam following intravenous midazolam injection (1.2 mg/kg IV) alone or in the presence of flumazenil (0.05 mg/kg IV) in 13 adult New-Zealand white-Californian cross rabbits. $t_{1/2}$: terminal elimination half-life; T_{max}: time at maximal plasma concentration; C_{max}: maximal plasma concentration; AUC_{0-t}: area under the curve of plasma concentration over time from time 0 to final timepoint.

		1-OH-midazolam alone (n = 13)		1-OH-midazolam with flumazenil (n = 13)		Paired t test
Parameters	Units	Mean value	95% CI	Mean value	95%CI	P value
$t_{1/2}$	min	97.9	63.8–132.0	84.7	61.9–107.4	0.48
T_{max}	min	17.4	9.7–25.1	20.8	15.9–25.8	0.43
C_{max}	ng/mL	42.9	31.6–54.2	40.6	29.1–52.2	0.76
AUC_{0-t}	ng·min/mL	2283	1662–2904	2200	1572–2828	0.84

Table 3. Pharmacokinetic parameters of flumazenil (0.05 mg/kg IV) in 13 adult New-Zealand white-Californian cross rabbits. $t_{1/2}$: terminal elimination half-life; T_{max}: time at maximal plasma concentration; C_0: extrapolated plasma concentration at time 0; AUC_{0-t}: area under the curve of plasma concentration over time from time 0 to final timepoint; $AUC_{0-\infty}$: area under the curve of plasma concentration over time from time 0 to infinity; MRT: mean residence time from time; Cl: clearance; V_{ss}: volume of distribution at steady state.

		Flumazenil (n = 13)	
Parameters	Units	Mean value	95%CI
$t_{1/2}$	min	26.3	23.3–29.3
C_0	ng/mL	195.2	158.6–231.8
AUC_{0-t}	ng·min/mL	2248	2011–2484
$AUC_{0-\infty}$	ng·min/mL	2776	2410–3143
MRT	min	34.03	30.00–38.07
Cl	mL/min/kg	18.74	16.47–21.00
V_{ss}	L/kg	0.6262	0.5460–0.7065

Discussion

To our knowledge, this is the first study describing the combined PK-PD parameters of IV FLU used to antagonize midazolam in rabbits. The FLU PK data (Table 3) reported in the current study are similar to those found in the existing literature in rabbits.[41] However, the PK values obtained here for FLU were measured in the presence of midazolam. Although FLU-midazolam PK interactions have not been reported in other species[31] and we did not observe an effect of FLU on midazolam PK, we cannot exclude this possibility. One group investigated the PK of a constant rate infusion (CRI) of FLU (1 mg/kg/hr IV) in 10 New Zealand white rabbits.[41] The reported terminal half-life (12 to 18 min) was lower than that observed here, due at least in part to a higher clearance in the previous study (approximately 35 to 40 mL/min/kg, which is nearly double the value we report). The previous study also found that theophylline inhibited FLU clearance, increasing the half-life of FLU to 28 to 42 min. Differences in PK modeling, administration method (IV bolus compared with CRI), or variability in study populations could also contribute to the differences observed.

FLU is currently only licensed for IV use in humans.[40] We used the IV route in the current to avoid the influence of absorption on efficacy and to facilitate direct comparison with human data. Another group reported a V_{ss} of 1.1 L/kg in humans, somewhat higher than the 0.6 L/kg observed in the rabbits in our study (Table 3). However, Cl was 16.3 mL/min/kg in humans, compared with 18.7 mL/min/kg reported here. The result is a half-life of approximately 60 min in humans, compared with 26 min in rabbits in our study.

Other routes of FLU administration have also been investigated. Oral (PO) administration results in a considerable first-pass metabolism in the liver, decreasing bioavailability to 16%, with a peak effect occurring 20 to 90 min after administration.[43] A comparison of IV, sublingual, intramuscular and rectal (PR) administration of FLU (0.02 mg/kg; 0.1 mg/kg for PR) to reverse midazolam-induced respiratory depression in mixed breed dogs reported no PK data and limited PD data were reported; however, the onset of action was the fastest after IV administration.[27] In another canine study, intralingual and oral submucosal administration of FLU (0.01 mg/kg) resulted in a similar peak plasma concentration as did IV injection,[50] but the time to peak concentrations was longer after intralingual and submucosal administration. At 30 min after administration, plasma concentrations for both intralingual and submucosal routes were higher than with IV administration.[50] Intraperitoneal and subcutaneous routes of administration are described in preclinical animal models reporting partial antagonism of benzodiazepine agonists.[32,35] However, to our knowledge, no PK investigations have been reported for these 2 alternative routes of administration. Therefore, further PK PD data are

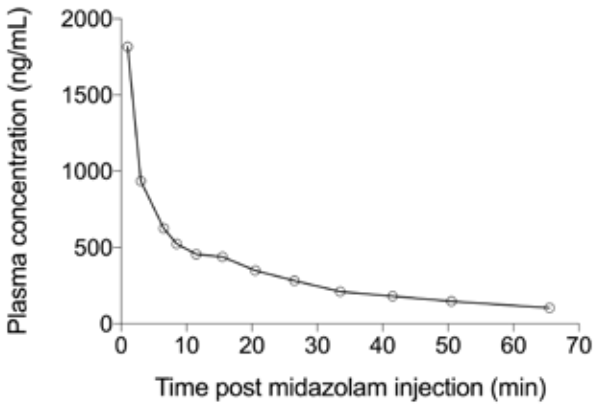

Figure 3. Example of observed plasma midazolam concentrations over time following an intravenous bolus of midazolam (1.2 mg/kg) in a New-Zealand white-Californian cross rabbit (ID: rabbit1arm1). Circles indicate arterial sampling times.

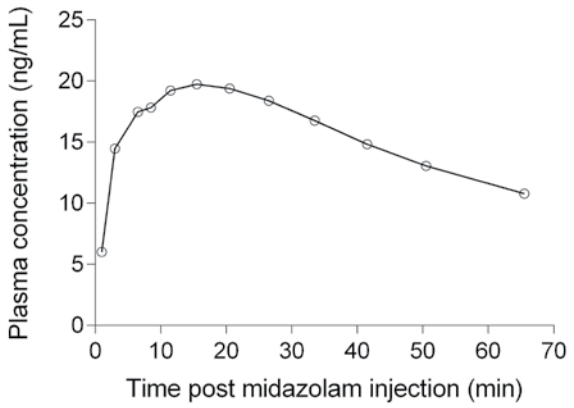

Figure 4. Example of observed plasma 1-OH-midazolam concentrations over time following an intravenous bolus of midazolam (1.2 mg/kg) in a New-Zealand white-Californian cross rabbit (ID: rabbit1arm1). Circles indicate arterial sampling times.

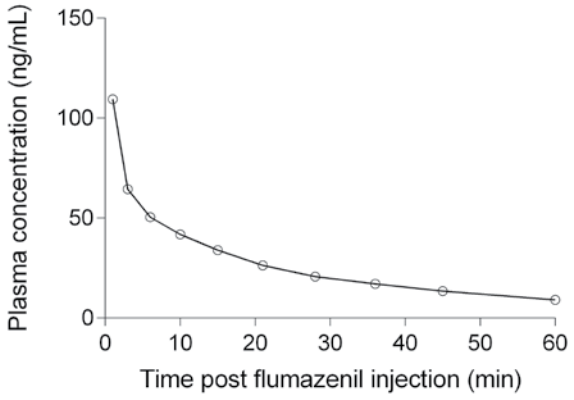

Figure 5. Example of observed plasma flumazenil concentrations over time following an intravenous bolus of flumazenil (0.05 mg/kg) in a New-Zealand white-Californian cross rabbit (ID: rabbit1arm1). Circles indicate arterial sampling times.

required to elucidate dosing guidelines using alternative routes of administration in animals.

The PK of midazolam in humans is substantially different than that of rabbits.[48] The half-life in humans is approximately 1.8 h, compared with approximately 30 min reported here. The V_d in humans and rabbits are similar at approximately 1.14 and 1.27 L/kg, respectively. This suggests that metabolism (reflected in the clearance) is much slower in humans (clearance of 5.5 mL/min/kg) than in rabbits (clearance of 35 mL/min/kg).

The PK of midazolam previously reported in rabbits is consistent with our results, with a half-life of 30 min and weight-normalized V_d and Cl of 0.83 L/kg and 19 mL/min/kg, respectively.[6] Important differences in dosage (0.35 compared with 1.2 mg/kg) could explain the small discrepancies in PK data between studies. Metabolism of midazolam by the liver leads to the production of an active metabolite, 1-OH-midazolam. This metabolite is produced in higher quantities after PO administration because of extensive first-pass metabolism,[3] whereas it merely reached 1/70th of the parent drug plasma concentration after IV administration in humans.[29] Although this metabolite has approximately half the activity of the parent drug and has a longer half-life, it is unlikely to have contributed to midazolam duration of action at the plasma concentrations reached in rabbits after IV administration, because the concentrations of midazolam were much greater than that of the metabolite.[17] FLU had no effect on the PK of midazolam or 1-OH-midazolam.

FLU (0.05 mg/kg IV) reliably reversed the sedative effects of midazolam (1.2 mg/kg IV) in less than 45 s, decreasing the duration of dorsal recumbency by a factor of 25. However, a return to lateral recumbency and LORR occurred after FLU administration. The time to return of lateral recumbency was longer after FLU administration and a significantly smaller proportion of rabbits were affected as compared with the SAL group. These results are consistent with previous studies reporting the efficacy of FLU (pharmaceutical compound code: Ro 15 to 1788) as a BZD antagonist in humans, rabbits, dogs, cats, rats, and mice.[4,13,38,49] However, these studies also report variable antagonist effect sizes, duration of action, and risks of resedation. The first reported use of FLU for reversal of midazolam-induced LORR in rats used an IV FLU dose of 3 mg/kg, which reduced LORR duration by a factor of 6.[9] This smaller effect size, as well as other differences reported in the literature assessing FLU antagonistic effects, could be explained by the agonist/antagonist dosage ratios, varying routes of administration, or varying outcomes of interest between studies.

In contrast to humans, dosage guidance for FLU is lacking for animals. The recommended FLU dosing strategy in humans is to titrate to effect, with an initial dose of 0.2 mg IV followed by increments of 0.1 mg IV, repeated every 60 s (maximal dose of 1 mg) until desired arousal is achieved. A typical total dose is between 0.3 and 0.6 mg.[40] This titrated administration decreases the likelihood of failed antagonism, which has been reported in 5% of reversals in humans and could reflect inadequate agonist/antagonist ratio at the site of action, based on individual variability.[36] One rabbit in our study did not achieve ReRR as expected after FLU despite plasma drug levels comparable to the rest of the sample population. Administration of FLU in veterinary patients is mostly reported as single injection. The appropriate agonist/antagonist dose ratio is important to achieve the desired level of arousal. The midazolam dose used here was chosen based on pilot data and associated with reliable LORR, whereas the FLU dose was based on clinical reports in the literature.[5,16,44] This resulted in an agonist/antagonist dose ratio of 1:24, approximately half that previously reported in dogs, where both sedation and muscle relaxation from midazolam (1 mg/kg, IV) were completely antagonized with FLU (0.08 mg/kg, IV; 1:13 ratio).[49] Although a lower agonist/

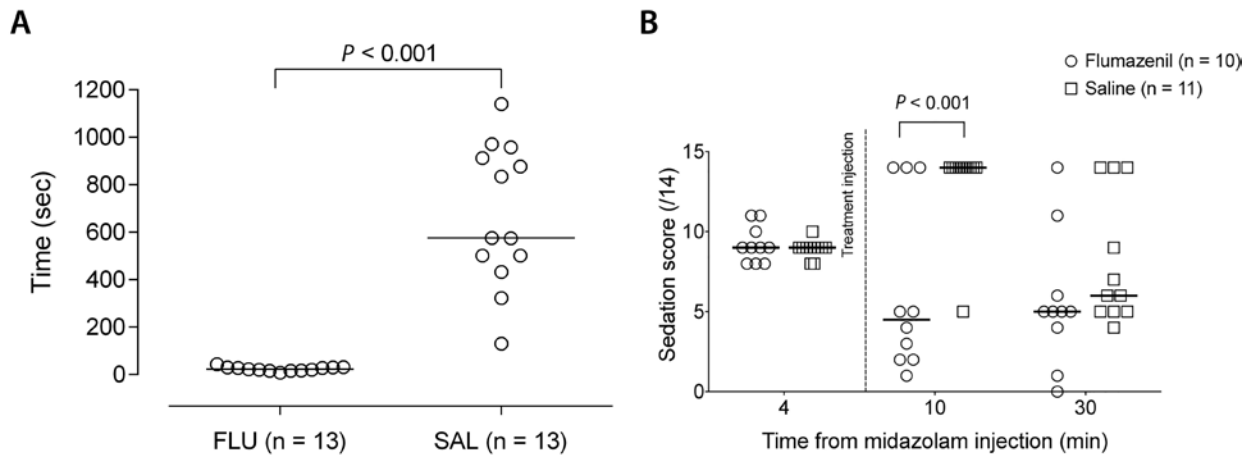

Figure 6. Time to return of righting reflex (ReRR) in 13 rabbits administered intravenous midazolam (1.2 mg/kg) followed by intravenous flumazenil (FLU, 0.05 mg/kg) or saline control (SAL, equal volume). Treatment (FLU/SAL) was administered 30 s after loss of righting reflex. Time to ReRR measured from FLU/SAL injection. Data are median (horizontal line) superimposed over individual data points. Time to ReRR significantly shorter in flumazenil group. (B) Scatter plot of sedation scale scores of rabbits administered intravenous midazolam (1.2 mg/kg) followed by intravenous flumazenil (FLU; 0.05 mg/kg) or saline control (SAL; equal volume). Treatment (FLU/SAL) was administered 30 s after loss of righting reflex and time of injection is represented by vertical broken line. Horizontal lines represent median. Sedation scores were significantly lower in FLU group at the 10 min postinjection time point.

antagonist ratio was used in our study, FLU proved effective in providing a rapid and reliable reversal of midazolam sedation. Importantly, resedation occurred in only approximately half of rabbits given FLU.

The risk of resedation in humans, resulting from a shorter clinical effect of FLU as compared with the BZD agonist, has been reported in anesthesia and critical care, but remains controversial. Studies evaluating FLU as an antidote for BZD intoxication likewise reported resedation; however the BZD dosages were higher than those used clinically.[1,39] Furthermore, the combination of BZD with other sedative agents (for example opioids) without the administration of proper reversal agents such as naloxone are important confounding factors preventing the accurate assessment of resedation after FLU administration.[21,28,37] Finally, absence of resedation was reported in multiple studies investigating the safety of using FLU to antagonize midazolam.[42,46,52]

The resedation reported after the use of FLU in this study has rarely been described in the veterinary literature. One group investigated the sedative effects of midazolam (1 mg/kg IM) followed by FLU (0.08 mg/kg IM) in ball pythons (*Python regius*). Signs of resedation were reported 3 h after reversal in all snakes (n = 9), although the level of sedation and the presence of adverse effects were not described.[33] Considering that midazolam is extensively bound to plasma protein (96%) in comparison with FLU (50%), elimination of the latter could be more rapid, a proposed explanation offered in some reports of resedation.[2,28] However, this explanation fails to explain the resedation we observed, as FLU and midazolam PK profiles were similar ($t_{1/2}$ of 25 to 30 min). Therefore, the shorter duration of BZD antagonism leading to resedation in rabbits could arise from inappropriate agonist/antagonist ratios (that is, too low a dose of FLU) or from the difference in free fraction of FLU at the site of effect (the brain). The rate and extent of drug delivery/elimination to/from the brain is affected by multiple factors that vary between species.[25] A good example of this is the blood-brain barrier efflux transporter P-glycoprotein resulting in faster elimination of FLU in rodents compared with humans.[19,20] PK-PD studies in rabbits investigating FLU uptake and elimination from the brain are warranted to elucidate the mechanism(s) underlying the duration of FLU action.

The delayed time to return of lateral recumbency after FLU administration (as compared with SAL) suggests FLU initially prevented the effects of midazolam, but eventually dropped below a concentration that was effective. Our approach of administering FLU immediately after the onset of midazolam effects would not be normal clinical practice. That is, we would only be reversing midazolam after the clinical procedure was completed, which could last anywhere from a few minutes to much longer in the case of a significant or complex surgical procedure. In those clinical situations, resedation may not have been observed, because of the longer time periods between administration of midazolam and the administration of FLU.

If resedation is a clinical issue, the problem could be addressed in 2 possible ways, neither of which were explored in this study. The first would be to increase the initial dose of FLU. Doses of up to 0.1 mg/kg have been given to individual rabbits, although this dosage has not been studied systematically.[5] Doubling the dose would be expected to extend the time of therapeutic efficacy by one half-life, or approximately 25 min. An alternative would be to administer a second dose of FLU. Given that the median time to a second loss of right reflex with FLU was about 19.8 min, and the median FLU concentration was 57.4 ng/mL, it would appear to be safe to administer a second dose of 0.05 mg/kg FLU. This is consistent with the recommendation in human patients, where repeat doses may be administered at 20-min intervals in the event of resedation.[40] Given the PK of midazolam in rabbits, the need to repeat FLU more than once appears unlikely, unless an unusually high dose of midazolam was administered. However, neither of these options were assessed in this study and are suggested based solely on the PK profile of FLU.

The absence of adverse cardiovascular effects from FLU are consistent with previous studies in rabbits.[8,45] Midazolam has a direct effect on contractility and systemic vascular resistance, potentially explaining the slow decrease in MAP over time in both treatment groups.[47]

The sedation scale used in this study was adapted from a validated canine sedation scale.[51] The modified scale was able

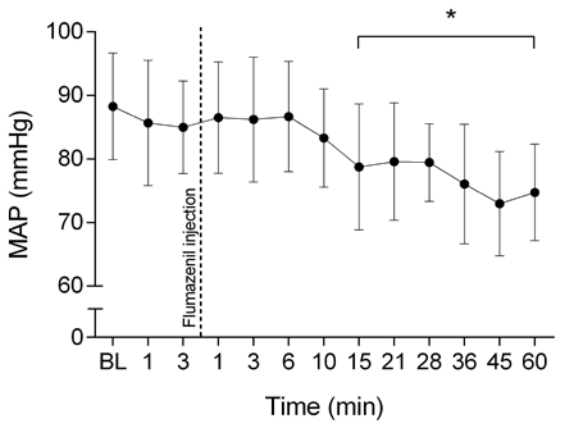

Figure 7. Mean arterial blood pressure (MAP) of rabbits administered intravenous midazolam (1.2 mg/kg) followed by intravenous flumazenil (0.05 mg/kg). Flumazenil was administered 30 s after loss of righting reflex and is represented by vertical broken line. Data are mean ± SD. MAP was lower than baseline (BL) at each timepoint, beginning 15 min post treatment injection ($P < 0.001$).

to detect a difference in sedation levels after SAL and FLU administration. This finding gives the scale construct validity, although further work is required to confirm this scale with other sedation protocols and to assess its reliability.

In addition to the rabbit that did not respond to FLU administration, another excluded rabbit never achieved LORR even after midazolam injection. Both rabbits had plasma concentrations for midazolam and FLU that were similar to the sample population mean, suggesting that the potential for individual variability reported in this species[2] is most likely related to pharmacodynamic, rather than pharmacokinetic differences. However, differences in brain concentrations cannot be discounted based on the data presented here. Five rabbits required a longer time to achieve LORR (7.5 to 10 min after midazolam injection). These rabbits were not excluded, as all timings were corrected to when drugs where administered and individual timepoints were used. Excluding these rabbits would assume that PK-PD relationship was similar across all rabbits, which does not reflect the data presented here.

A limitation of this study was the single-dose and route of administration used to determine the PK-PD profile of FLU in rabbits. Although this limits the generalizability of the results, these data remain the first available PK-PD profile of FLU and midazolam coadministration in rabbits and could be the basis for further investigations. The decision to perform a PK-PD study using standard methodology was based on the goal of generating an evidence-base for clinical application. Another limitation was not using a midazolam CRI to confirm the plasma concentration at which the effect of FLU begins or wears off because both the concentration of FLU and midazolam were changing over time. As the plasma concentration of both FLU and midazolam decrease, it is difficult to isolate the sole effect of FLU plasma concentration on its duration of effect. Furthermore, without any assessment of the effect site (brain) concentration, the relationship between plasma concentration and brain concentration is unknown. However, the administration of midazolam as a single IV bolus reflects clinical practice.[3,22] This single IV bolus approach is more conservative, as the risk of resedation decreases with a longer delay between agonist and antagonist injection. A final limitation of this study was the absence of objective quantification of the effects of resedation. As described, all rabbits completed the study without any observable adverse effect; nonetheless, objective measures of ventilation or oxygenation by blood gas analysis would provide a more accurate assessment of the risk associated with resedation.

In conclusion, FLU (0.05 mg/kg, IV) antagonizes the sedative effects of midazolam administered intravenously at 1.2 mg/kg in rabbits. However, a risk of resedation at approximately 20 min after FLU injection warrants continued monitoring in the recovery phase. Further research is necessary to determine if alternative dosing strategies of FLU in rabbits can be used to avoid this resedation event.

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