Comparative pharmacokinetics of submucosal *vs.* intravenous flumazenil (Romazicon®) in an animal model

Faith M. Oliver, DDS Trevor W. Sweatman, PhD John H. Unkel, DDS, MPA Michael A. Kahn, DDS
Mildred M. Randolph, DVM Kristopher L. Arheart, EdD Timothy D. Mandrell, DVM

Dr. Oliver is a resident, Pediatric Dentistry; Dr. Sweatman is an associate professor, Pharmacology; Dr. Unkel is an associate professor and postgraduate program director, Pediatric Dentistry; Dr. Kahn is a professor, Biologic & Diagnostic Sciences; Dr. Randolph is a veterinarian, Comparative Medicine; Dr. Arheart is an assistant professor, Preventive Medicine; and Dr. Mandrell is an associate professor, Comparative Medicine, they are all at the University of Tennessee. Correspond with Dr. Unkel at junkel@utmem.edu

Abstract

Purpose: This study was performed to determine the bioavailability and local tissue toxicological safety of flumazenil (Romazicon®) when administered by oral submucosal (SM) as opposed to intravenous (IV) injection.

Methods: Six dogs each received SM flumazenil (0.2 mg), and their serum was collected at predetermined time intervals (0-2 h) and frozen (-70°C). Seven days later, the dogs received an identical dose of IV flumazenil, and serum samples were again collected, as above. Comparative quantitation of flumazenil levels (IV vs. SM) was made using a sensitive HPLC assay (UV detection). Direct/local drug toxicity was visually scored by unbiased raters of color photographs (test and control mucosa) taken at 1, 2, and 7 days following SM flumazenil injection. An oral pathologist examined slides processed from control and treatment tissues (hematoxylin and eosin staining) taken (punch biopsy) 1 week following SM injection to compare with direct clinical scores.

Results: Serum flumazenil levels reached a plateau (8.5 ± 1.5 ng/mL, mean ± SD) within 4 min of SM drug injection and declined thereafter to ~2 ng/mL by 2 h. Bioavailability of SM flumazenil was 101 ± 14%, based upon measuring the area under the serum concentration-time curves over 1.5 h (AUC 0-1.5 h, SM vs. IV drug). Thus, serum drug levels following SM drug administration were broadly comparable to those obtained during the elimination phase of corresponding IV drug delivery. Regarding drug tissue toxicity, no evidence of direct drug toxicity was observed by unbiased raters of color photographs (test and control mucosa) taken at 1, 2, and 7 days following SM flumazenil injection. Following pathologic review, no difference was seen in the degree of inflammation between treatment and control tissue.

Conclusion: At the quantity and concentration used, SM drug flumazenil administration appears to be both a safe and a viable alternative to bolus IV drug delivery and worthy of further investigation. (Pediatr Dent 22:489-493, 2000)

Intravenous (IV) benzodiazepines such as diazepam (Valium®) and midazolam (Versed®) have been used for more than 10 years both as components of general anesthesia and, where appropriate, for conscious sedation during endoscopy, urology, cardiology, and outpatient surgery. These drugs act by enhancing gamma-amino butyric acid (GABA) binding to specific receptors located in the central nervous system (CNS), thereby opening chloride ion channels. This results in post-synaptic membrane hyperpolarization and reduced cortical stimulation *via* the lower brain centers. Traditional endpoints for benzodiazepine-induced conscious sedation (ptosis, dysarthria, and drowsiness) are very close to an hypnotic state in which the patient is unresponsive to verbal command.[1,2] More recently, anxiolysis, amnesia, and patient cooperation have been used as measures of drug effectiveness.[3] With regard to dentistry, the intranasal (IN: 0.2-0.4 mg/kg) or oral (PO: up to 0.6-0.7 mg/kg) administration of the short-acting water-soluble benzodiazepine midazolam is increasingly common in the practice of pediatric sedation. Not only does midazolam produce a more rapid sedative effect than diazepam, but also the lack of pharmacologically-active midazolam metabolites provides for a more rapid recovery with this agent, typically in 45-60 min.[4] In general, midazolam is well tolerated with few significant hemodynamic effects. However, either through inappropriate IV dosing (dose- and speed-sensitive) or by virtue of an iatrogenic response, midazolam, like the other benzodiazepines, is capable of producing life-threatening respiratory depression and apnea which may require pharmacologic intervention.

In contrast to other sedative drugs, one significant advantage of benzodiazepine-induced sedation is the clinical availability of a specific and potent antagonist, flumazenil (Romazicon®). This imidobenzodiazepine derivative whose pharmacology as a benzodiazepine antagonist was first reported in a series of publications almost two decades ago,[5-8] does not itself possess intrinsic sedative activity at normal therapeutic doses[3] and does not change the benzodiazepine pharmacokinetics when the two agents are present in the circulation at clinical concentrations.[9,10] Flumazenil has a very high specificity for the CNS benzodiazepine receptors where displacement of the benzodiazepine results in closure of the chloride channels, membrane re-stabilization, and rapid recovery of respiratory and cognitive function. Indeed, such is the receptor specificity which this drug exhibits that radiolabeled (C^{11})-flumazenil has been employed during *in vivo* benzodiazepine receptor binding studies[11] and more recently as a means to study the reduction in CNS benzodiazepine receptor density and function during epilepsy.[12]

Flumazenil is pharmacologically effective when administered PO; however, the antagonist undergoes extensive first-pass hepatic metabolism, with a bioavailability of approxi-

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mately 15%.[13] Flumazenil has also proven effective in reversing benzodiazepine sedation when administered rectally[14] and by endotracheal instillation,[15] thereby indicating that this lipophilic agent has the capacity to readily traverse biological membranes. Nevertheless, flumazenil is presently approved only for IV administration. In humans, IV flumazenil doses of 0.5 mg or less produce recovery of cognitive function in a matter of seconds, i.e., one circulation time from arm to brain.

With regard to metabolism, less than 0.2% of an IV dose of flumazenil is recovered unchanged in the urine,[16, 17] the principal excretory product being a glucuronide conjugate of flumazenil "acid." The circulating metabolic products of flumazenil consist principally of cleaved products arising from esterase activity and/or N-demethylation; none of these products possesses biological activity. As a result both of its extensive and rapid hepatic extraction and through the action of ubiquitous esterase enzymes, flumazenil has a short half-life in serum, in the order of 45-80 min; [18-21] by contrast, the serum half life of midazolam is 1.5-3 h.[22] Therefore, the potential for the re-emergence of residual benzodiazepine sedation exists. Although a single IV dose of flumazenil provides effective benzodiazepine antagonism for about 15 min, repeated doses of flumazenil may be necessary to maintain an antagonistic effect, most especially in patients who have received long-acting benzodiazepines and with intentional drug overdose. Nonetheless, with respect to normal clinical midazolam doses, reversal of respiratory depression is rapidly accomplished with a single flumazenil administration of 0.5 mg or less. In terms of safety, the earlier reports of convulsions or seizures arising from flumazenil administration[23-25] are now recognized as resulting from rebound CNS stimulation following benzodiazepine receptor down regulation. This is a result of either chronic benzodiazepine-induced or natural pathological changes arising from disease processes. Indeed, recent studies have employed flumazenil pre-surgically to induce and thereby delineate epileptic seizure foci in patients with medically intractable localization-related epilepsies.[26] Regardless, in normal individuals flumazenil possesses a wide therapeutic safety margin and a low risk for toxicity, even when an accidental flumazenil overdose is given.[18, 19] Currently, flumazenil is indicated for the partial or complete reversal of the sedative effects of benzodiazepines in cases where general anesthesia has been induced and/or maintained with benzodiazepines for diagnostic and therapeutic procedures[20] and for patients with benzodiazepine intoxication resulting from iatrogenic overdose.[21, 27] Recommended clinical dosing for flumazenil is 0.2 mg, followed by incremental 0.1-mg doses up to 1 mg, until the desired endpoint is achieved; however, some anesthetists prefer to employ 0.5 mg immediately in healthy individuals.[27] Reversal of sedation with IV flumazenil is generally evident within 1 to 2 min after administering a dose of 0.2 mg and the drug reaches maximal effect in about 5 min.[17,28] Antagonist is also used to mediate depth of sedation induced by benzodiazepines. Titration of the reversal agent permits sedation; however, the patient is rousable upon command.[21] For these indications, flumazenil is administered by IV injection.

In the context of pediatric dental sedation however, routine use of IV drugs is unusual and, as noted above, midazolam is customarily administered PO or IN. Therefore, in those rare situations where the benzodiazepine produces an iatrogenic response, immediate IV access is not generally available. In such circumstances, the SM route, which is routinely utilized for the delivery of dental local anesthetics, would seem a desirable alternative. Therefore, the purpose of our study was to investigate the practicality of this rapid dosing technique by determining the bioavailability and toxicological safety of flumazenil when administered by SM injection.

Methods

Model description

The dog was chosen as an animal model based upon comparable body weight to children (20-25 kg) and the ease of oral access in this species. All experiments with dogs were conducted under protocols approved by the institutional ACUC review process. At no time were the animals subjected to painful procedures. Overall supervision of the animal experiments was the responsibility of the Department of Comparative Medicine.

Animal studies

Six anesthetized (isoflurane) male dogs (20-25 kg, overnight-fasted) each received a single injected SM dose of flumazenil (0.2 mg) in the mucobuccal fold mesial to the third-fourth premolar (an area comparable to the first-second premolar mucobuccal fold used by others in clinical studies).[29] Immediately before (0 time) and at various times (1, 2, 3, 4, 6, 10, 15, 20, 30, 60, 90 and 120 min) following drug injection, blood samples (5.0 mL) were obtained from an indwelling cephalic vein catheter (20G 1.25 in). Catheter patency was maintained by back flushing with heparinized saline. No additional medications were administered to the dogs, and no physiological effects of flumazenil administration were observed over the course of the study. Blood samples were transferred into sterile vacutainers, which were immediately placed on ice. Following centrifugation, the serum was transferred to labeled vials and immediately frozen (-70°C) pending analysis. Selection of these sample collection times was based upon published pharmacokinetic data indicating a short plasma half-life and rapid decline in detectable blood levels of flumazenil.[17]

One week following the SM drug dose and using identical experimental conditions to those described above, each dog received a second flumazenil (0.2 mg) dose by bolus IV injection via the cephalic vein. Blood samples were obtained from the contralateral cephalic vein and processed in a manner identical to that described above. Based upon current knowledge of flumazenil pharmacology, namely the short plasma half-life of this drug, this 7-day interval was considered more than sufficient to eliminate any order effect between the two treatments. In addition to the blood samples, punch biopsy tissue samples of the earlier SM injection sites and contralateral (control) tissue sites were obtained at this time and placed in labeled containers of 10% buffered neutral formalin for subsequent histologic examination.

Animals were allowed to recover consciousness spontaneously at the conclusion of the 2-h serum-sample collection period and were returned to their pens with routine access to food and water. Approximately 24, 48, and 168 h following SM drug injection, color photographs were taken of the flumazenil injection sites and corresponding contralateral (control) mucosal areas. No physiological adverse effects of flumazenil were evident, either by IV or SM administration.

Analytical studies

Quantitation of flumazenil in plasma was made using a reversed-phase HPLC assay that was described previously.[9]

Table 1. Estimates of Observed Inflammation and Rater Agreement

	Percentage of Inflammation		Percent Rater Agreement	
Time	N	Estimate	N	Estimate
Day 1	100 = 5 dogs x 20 raters	0.06 (0.02 - 0.11)	190 rater pairs	89 (87 – 90)
Day 2	100 = 5 dogs x 20 raters	0.06 (0.02 - 0.11)	190 rater pairs	89 (87 – 90)
Day 7	120 = 6 dogs x 20 raters	0.04 (0.02 - 0.08)	190 rater pairs	92 (91 – 93)
Over All	320 = 100 + 100 + 120	0.05 (0.03 - 0.08)	570 rater pairs	90 (88 – 90)

Note: The pictures for dog 6 were unusable for days 1 and 2.

Briefly, duplicate aliquots (0.5mL) of thawed plasma samples were buffered (phosphate) to pH 9.1 with the addition of 20 µL of a methanolic solution of flurazepam (Dalmane®) as internal standard. Aqueous phases were then subjected to a liquid-liquid extraction (2 x 7 mL) with ice-cold dichloromethane/diethyl ether (4:6, v/v). For each sample, the combined organic phase was evaporated under dry nitrogen at 37°C. Dried samples were either reconstituted immediately or were frozen (-70°C) for subsequent analysis. Samples were reconstituted in methanol (80 µL) and were placed in sealed disposable glass inserts (250 µL) in an injector tray for automated HPLC analysis, which was conducted using a Waters Associates (Milford, MA) Nova-Pak™ C_{18} reversed-phase column (4 µm; 10 cm x 5 mm i.d.) with a mobile phase of HPLC-grade acetonitrile in sodium phosphate buffer (0.04M, pH 7.2; containing 0.1% triethylamine) at 1.5 mL/min. The elution gradient changed from initial conditions (10%) to 22% acetonitrile in 13 min to elute the flumazenil (retention time, 15.3 min), followed by an increase to 63% acetonitrile over the ensuing 12 min, to elute the less polar internal standard (retention time, 22.3 min). Peak detection was made by a Waters Associates (Milford, MA.) Model 2487 dual wavelength UV detector set at 243 nm. Calibration curves were constructed according to standard techniques using blank human serum (0.5 mL) spiked with known concentrations of flumazenil (5, 10, and 40 ng/mL, in duplicate) and processed in parallel with test samples. Quantitation was made by interpolation of unknown peak ratio (*vs.* internal standard) values into the standard calibration curves, and the values were reported as the mean of duplicate analyses.

This flumazenil assay was both sensitive (limit of quantitation, 0.5 ng/mL) and reproducible with coefficient of variability values of 3.4%, 6.3%, and 7.0% for 40 ng/mL, 10 ng/mL, and 5 ng/mL spiked standards, respectively (N=10). The efficiency of flumazenil standard (5ng-40 ng/mL) extraction from blank human serum was 100 ± 5%.

Histology studies

Tissue specimens were submitted to a University Oral and Maxillofacial Pathology Lab in coded containers to enable unbiased interpretation. For each animal, the lab prepared microscopic slides each of 5-µm tissue thickness and stained them with hematoxylin and eosin. The lab reported any tissue changes noted on each slide including, but not limited to, polymorphonuclear neutrophils, macrophages, plasma cells, and B and T lymphocytes.

Data analysis

For each animal, Graph Pad® (San Diego, CA.) software calculated the area under the serum flumazenil concentration-time-curves up to 1.5 h following IV and SM drug administration (AUC 0-1.5 h, ng/mL-hr). From this information, bioavailability was calculated as (AUC_{SM} /AUC_{IV}) x 100%.

Twenty unbiased raters examined unmarked photographs of the injection and control tissue areas of each of the 6 dogs on days 1, 2, and 7 and reported whether or not clinical changes were present. Incidence of inflammation was reported as a mean percentage of positive findings over all dogs and raters for each day and over all 3 days. Rater agreement was expressed as a mean percent agreement over all dogs for all 190 pairs of raters for each day and over all 3 days. Incidence and rater agreement data were subjected to a boostrap analysis to calculate 95% confidence bounds for the estimates. The boostrap procedure is a computer-intensive technique that redraws samples randomly from the original sample with replacement.[30] Bootstraping allows the study of the distribution of sample statistics that might otherwise be too complicated to consider. The technique, which requires simple calculations, involved drawing repeated random samples with replacement from the actual data distribution and then building a distribution for a statistic by calculating a value of the statistic for each sample.[31] In this study, the analysis was appropriate to use to determine the 95% confidence bounds of the incidence of inflammation and the extent of agreement amongst the raters. Ten thousand repeated samples were made for each confidence interval calculation. For inflammation, the samples were taken from the set of ratings of each dog by all raters, and for rater agreement the sample was taken from the agreement of all possible pairs of raters. A descriptive analysis was used to report the observation of histological tissue changes.

Results

The serum flumazenil profiles following IV and SM drug administration are shown in Figure 1. As expected, peak serum levels of IV drug occurred immediately (23.4 ± 4.9 ng/mL at 1 min), with concentrations declining thereafter in a bi-exponential manner to < 5 ng/mL by 0.25 h. By contrast, peak serum levels of SM flumazenil (8.5 ±1.5 ng/mL) occurred at ~4 min. Thereafter, the decline in drug level was comparable to that observed for IV administration, although absolute levels remained marginally higher through 1 h, indicating perhaps additional drug absorption from the mucosal site during this period. Bioavailability of SM flumazenil was excellent at 100 ± 14%. To put these serum data in an appropriate clinical perspective, levels of flumazenil >5 ng/mL are sufficient to reverse benzodiazepine sedation.[10]

The amount of inflammation observed by the unbiased raters in the photographs of the injection and control mucosal areas was minimal (Table 1). Agreement amongst the raters as to the presence or absence of inflammation was excellent (Table 1). The results of the histological examination of the

Table 2. Histological Observations of Submucosal Flumazenil Injection Sites (Test) *vs.* Contralateral (Control) Tissue Samples

Number of samples scored		Pathology Observation
Test	Control	
-	1	no inflammatory cellular infiltrate
1	1	scant lymphocytes
3	-	scant lymphocytes and some mast cells
-	1	sparse lymphocytes and occasional plasma cells
-	3	mild lymphocytes, perivascular plasma cells
2	-	lymphocytic, plasmacytic

N=6 for Test and Control samples

treatment/control punch-biopsy tissue samples are shown in Table 2. No clinically significant difference is evident in the treatment *vs.* control histology.

Discussion

The efficacy and pharmacokinetics of IV flumazenil have been previously evaluated in rats, mice, cats, and dogs at doses ranging from 0.3-30 mg/kg; no studies of SM flumazenil have been reported. With regard to published clinical studies, peak plasma levels of flumazenil and the time at which they occur is dependent upon both the dose and the speed of drug administration. Thus, peak levels occur in 2 min or 5 min following a 1 or 20-mg[11] IV dose.[32, 20] In the present study, the finding of the peak IV drug concentration in the first serum sample obtained at 1 min reflects the smaller dose and volume administered. Thereafter, clinical studies show that flumazenil behaves according to a 2-compartment pharmacokinetic model with a rapid distribution half-life, generally in the order of 5 min or less,[32] followed by an elimination half-life of about 1 h.[21, 32] Significantly, these kinetic values remain unaffected by age.[21] With regard to the present study, bioavailability was calculated to allow comparison of SM with IV administration and a formal pharmacokinetic analysis was not performed. Nevertheless, the pattern of serum flumazenil achieved following IV drug dosing at a clinical dose of 0.2 mg is consistent with previously reported clinical models.

Intraoral submucosal injection of flumazenil (Romazicon®, as an IV formulation) appears to be a viable concept based upon the following findings. The drug is rapidly and completely absorbed into the systemic circulation, as evidenced by comparable serum concentrations to those obtained by IV administration. It should be emphasized that this was a study that used absolute drug levels as an endpoint and did not take into account their pharmacologic effectiveness. Nonetheless, serum flumazenil concentrations of ~5ng/mL, as seen with SM drug delivery, are consistent with clinical reversal of sedation.[10] However, our experiment relied upon a clinically formulated drug (0.2 mg/2 mL) that, based upon the consideration of a 2-mL maximum SM volume, provided no opportunity to increase drug dose; this volume is comparable to the commonly administered volume of a single dental anesthetic carpule. For consistency, the drug was always injected in the mucobuccal fold mesial to the third-fourth premolar.[29] The contralateral side of the maxillary oral vestibule was employed as a control in the histology studies, so additional drug administration here was not possible.

Further investigation is required to determine if a reformulated (more concentrated) drug administered in a smaller volume into the same injection site or opposing jaw mucosa will achieve viable flumazenil redosing. A reformulated drug may also be beneficial in providing more information regarding flumazenil adsorption from the SM site. For example, in the present study the effect of dose volume on the kinetics of drug absorption was not investigated; would a lower dose volume provide for a shorter or a more extended drug release? Certainly, administration of the half the volume (1 mL) using drug formulated at twice the present concentration, i.e. 0.4 mg/mL, would still remain well below the recommended 1 mg total for IV drug dosing and beneath the aforementioned 0.5 mg IV bolus administration advocated in some clinical situations.

Submucosal drug delivery is potentially a means for the pediatric dentist to provide an immediate pharmacologic response to unanticipated benzodiazepine oversedation without recourse to IV access. Depending on the individual circumstances relative to the benzodiazepine, route of administration and dose employed, this technique may be sufficient in and of itself in maintaining the patient's respiratory function. Reemergence of sedation, albeit at a reduced intensity, could theoretically occur; however, this is unlikely given the doses and routes of midazolam administration commonly employed in pediatric dentistry. Regardless, the ability of the pediatric dentist to provide a rapid and immediate mediation of benzodiazepine-induced apnea would allow a window of time for IV access in such a patient to be established. One should not assume that SM delivery is preferential to IV titration in order to provide minute to minute control over depth of sedation.

Relative to the question of potential toxicity of the drug or coformulated materials, no evidence of clinical change could be observed in the mucosal surface in the week following drug

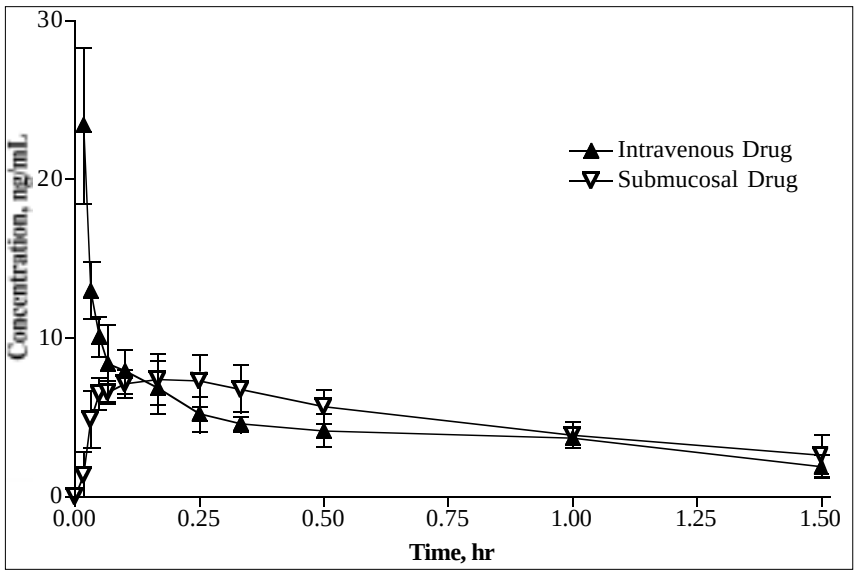

Fig 1. Comparative dog serum flumazenil concentrations (mean ± SD) following intravenous vs. submucosal drug administration.

injection. Moreover, upon histological examination, the degree of inflammation in test samples was not discernibly different from that of control tissue. Therefore, it can be assumed that SM delivery of IV formulated flumazenil is well tolerated at the quantities and concentrations used.

Conclusions

1) SM injection of flumazenil appears to be a reliable and viable alternative to immediate IV administration for reversing benzodiazepine sedation.
2) The data suggest that a more concentrated form of flumazenil (e.g. 0.4 mg/mL) might be developed to allow the attainment of serum drug levels >5 ng/mL for a more extended period. Such a formulation could reduce the potential for sedation by long-acting benzodiazepine(s) to re-emerge.
3) Additional studies of SM flumazenil appear to be warranted.

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