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A Comparison of the Physical Dependence Inducing Properties of Flunitrazepam and Diazepam

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SLOAN, J. W., W. R. MARTIN AND E. P. WALA. *A comparison of the physical dependence inducing properties of flunitrazepam and diazepam.* PHARMACOL BIOCHEM BEHAV **39**(2) 395–405, 1991.—Dogs dosed chronically (4–7 weeks) with oral flunitrazepam (7.6 mg/kg/day) or diazepam (24–36 mg/kg/day) administered in 4 equally divided doses had dose-related flumazenil precipitated benzodiazepine abstinence scale scores (BPAS) of comparable intensities despite the fact that plasma levels of flunitrazepam and its metabolites were much lower than nordiazepam levels in the diazepam-dependent dog. Both groups of dependent dogs had clonic and tonic-clonic seizures after oral and IV flumazenil. Precipitated abstinence signs persisted longer in the diazepam than in the flunitrazepam-dependent dogs. Differences in the pharmacokinetics of the drugs of dependence, their metabolites, and their interactions at receptor sites offer a partial explanation for the high level of dependence seen in the flunitrazepam dog. The finding that the estimated plasma free concentration of flunitrazepam and its metabolites is equal to or greater than that of diazepam and its metabolites together with the fact that flunitrazepam has a higher affinity for the benzodiazepine receptor than either diazepam, nordiazepam or oxazepam can explain why the intensity of the precipitated abstinence syndrome is comparable in flunitrazepam- and diazepam-dependent dogs. Although the flumazenil-induced precipitated abstinence syndromes observed in flunitrazepam- and diazepam-dependent dogs differed qualitatively they did not differ quantitatively. It is therefore concluded from these data that the doses of flunitrazepam and diazepam, chosen for producing comparable degrees of weight loss during dose escalation, did not differ in the degree of physical dependence that they produced in the dog.

Flunitrazepam dependence in dogs Diazepam dependence in dogs Precipitated abstinence Flumazenil
Flunitrazepam plasma and brain levels Diazepam plasma and brain levels
Chronic diazepam and plasma protein binding Chronic flunitrazepam and plasma protein binding

FLUNITRAZEPAM is a benzodiazepine which differs structurally from diazepam by the substitution of a nitro group in the 7 position and a fluorine at 2′ in the other phenol ring (Fig. 1). It has been used mainly as a nighttime hypnotic, and like diazepam, is used in surgery for the induction of anesthesia. Flunitrazepam is also a potent anxiolytic, but because of its strong hypnotic effect, diazepam is the drug of choice for daytime sedation (23). Since it is a strong hypnotic, flunitrazepam has been abused by opiate addicts for the relief of some of the signs of abstinence such as irritability and insomnia (5). Numerous clinical reports have shown that chronic treatment with either diazepam or flunitrazepam leads to a withdrawal syndrome including clonic and tonic-clonic convulsions (8,22). Unfortunately, most of these studies are confounded by the fact that the patients take a variety of other drugs along with the benzodiazepines. It is therefore important to assess the chronic effects of these drugs alone for their dependence-producing properties. Previous studies of diazepam and nordiazepam dependence in the dog have shown that chronic dosing to the point of liminal weight loss with these drugs can produce physical dependence characterized by a well-defined withdrawal abstinence syndrome (17,18) and a flumazenil-precipitated abstinence syndrome (17, 19, 21, 22). These syndromes are characterized by tremulousness, rigidity, twitches and jerks, myoclonus, hot foot walking, tachypnea, lip-licking, clonic convulsions, tonic-clonic convulsions and status epilepticus.

Both flunitrazepam and diazepam are widely prescribed for chronic use and are also popular adjuncts for induction anesthesia. Flumazenil has been shown to improve emergence from flunitrazepam- or midazolam-induced general anesthesia in human subjects (1,12) and its use for this purpose has recently been approved by the FDA. It is therefore important to assess both the dependence-producing properties of benzodiazepines that are used chronically and in anesthesia as well as their interaction with benzodiazepine antagonists.

The purpose of this communication is to compare the ability of flunitrazepam to produce physical dependence with that of diazepam (as measured by the flumazenil-precipitated abstinence

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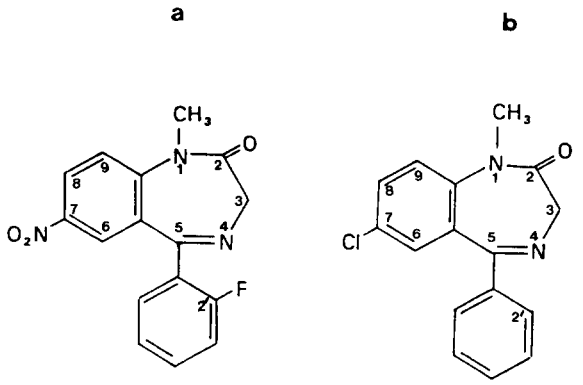

FIG. 1. Structures of (a) flunitrazepam and (b) diazepam.

syndrome) in dogs which have been dosed orally at comparable doses and to assess both quantitative and qualitative aspects of the syndromes.

METHOD

Subjects and Dosing Procedures

The methods employed in these dependence and precipitation studies have been previously described (19,22). In the present, study, six beagle-type dogs were made dependent on flunitrazepam or diazepam using an escalating dose schedule until an oral dose was achieved which produced weight loss. These doses were 19 mg (7.6 mg/kg/day) of flunitrazepam or 50, 60 or 90 mg [20 (1 dog), 24 (1 dog) or 36 (4 dogs) mg/kg/day] of diazepam. Each drug dose was administered orally in #4 gelatin capsules in equally divided doses approximately every 6 hours. The final doses of flunitrazepam and diazepam that were selected for stabilization produced an initial weight loss of between 0.2 and 1.1 kg (-0.5 ± 0.2 kg, $n=6$ for flunitrazepam and -0.4 ± 0.2, $n=6$ for diazepam). The doses of diazepam chosen produced comparable plasma levels of nordiazepam. Neither diazepam nor flunitrazepam produced any overt signs of sedation or motor incoordination during dose escalation or stabilization. Approximately three weeks were required to reach the stabilization dose and the dogs were held at this dose for at least two weeks before precipitation studies were initiated. Table 1 outlines the dosing parameters and the chronology of the precipitation experiments.

Precipitated Abstinence

Dogs were brought to the observation room 24 hours before the precipitation studies where they were deprived of food but not water at 1900. They received their maintenance dose of flunitrazepam or diazepam at 0700 on the day of the experiment. Body weight and rectal temperatures were measured just prior to the oral administration of flumazenil or a lactose placebo at 0800. They were then observed for signs of abstinence for four hours which were recorded on a standard observation sheet (17, 18, 20, 22, 30). Each dog was observed by the same observer (J.W.S. or W.R.M.) throughout these experiments. At the end of the four-hour observation period, body weight, rectal temperature and water intake were recorded for the flunitrazepam- and diazepam-dependent dogs. Two doses of flumazenil (2 and 6 mg/kg and a placebo) were administered to the flunitrazepam-dependent dogs and three doses of flumazenil (2, 6 and 18 mg/kg) were administered to the diazepam-dependent dogs at weekly intervals using a replicate block design. Both blocks (3×3), in which the doses were randomized, were conducted on a blind basis (observers) using a Latin Square design. All flunitrazepam-dependent dogs received 18 mg/kg doses of flumazenil and the diazepam-dependent dogs received a lactose placebo one week after the end of the replicate block experiment. Approximately 3 to 4 weeks later a second 18 mg/kg dose of flumazenil was administered to both flunitrazepam- and diazepam-dependent dogs, where only body temperature and seizure activity were recorded. The dogs were observed for signs of abstinence for observation periods which ended sequentially 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after the administration of flumazenil. Both a behavioral score and a benzodiazepine-precipitated abstinence score (BPAS) (22) were calculated from these observations. The behavioral states included in the behavioral score and their weights were: sitting, 1; standing, 2; walking, 3; exploring the cage, 5; prone, -2; and immobile for over 5 minutes, -5. The behavioral score was obtained for each epoch of the observation period by weighting the signs, obtaining the sum of these weighted values and dividing this sum by the number of signs comprising the total. The BPAS scale is a modification of previously developed abstinence scales (19) and consists of ten signs of abstinence (22). These signs and their assigned weights are: limb and neck tremors, 3; whole body tremors, 3; twitches and jerks, 1; hot foot walking, 2; rigid walking, 5; lip licking, 0.5; respiratory rate, 1; clonic seizures, 20; tonic-clonic seizures, 80; and status epilepticus, 100. The weighing values were assigned such that each sign made an approximately equal contribution to the scale score. The area under the time-action curve (AUC) for each dog and for each dose of flumazenil was determined by the trapezoidal rule. The BPAS score was then calculated by adding the weighted areas for each of these ten signs. All values are presented with the lactose placebo value subtracted except where indicated. These scores were used to determine dose-response lines which were analyzed using a two-way analysis of variance (dogs $\times$ doses) and the between doses variance was partitioned into regression and deviation from linearity variance. Dose-response relationships were compared and relative potencies calculated using an ANOVA for an incomplete block design (9).

TABLE 1

CHRONIC DOSING PARAMETERS FOR FLUNITRAZEPAM- AND DIAZEPAM-DEPENDENT DOGS (SEE THE METHOD SECTION FOR DETAILS)

Drug	No. of Dogs	Maintenance Dose (mg/kg/day)	Weeks to Reach Maintenance Dose	Weeks on Maintenance Dose: Crossover 1st ppt Week	Crossover Last ppt Week	IV ppt Week	Week Sacrificed
Flunitrazepam	6	7.6	3.0 ± 0	4.8 ± 0.1	7.5 ± 0.3	14.0 ± 0.2	14.8 ± 0.6
Diazepam	6	20, 24, 36	3.3 ± 0.5	4.1 ± 0.4	7.3 ± 0.3	14.2 ± 1.7	16.8 ± 1.9

When all of the oral precipitation and pharmacokinetic studies were completed, each flunitrazepam-dependent and 5 of the 6 diazepam-dependent dogs were transferred to an observation room and placed in a dog sling 1.5 to 3 hours after the morning stabilization dose of the drug of dependence. All flunitrazepam-dependent dogs and 3 of 6 diazepam-dependent dogs received an intravenous infusion of flumazenil (11 mg/ml) dissolved in absolute ethanol through a PE 20 polyethylene cannula whose tip was placed in the subclavian vein. The solution was administered at a rate of about 1 ml/min and was discontinued when a convulsion or extreme nuchal or limb rigidity was induced. Two diazepam-dependent dogs received an IV injection (~2 ml) of a 5% liposomal suspension of flumazenil (Hoffmann-La Roche). This suspension was not available until all of the flunitrazepam-dependent and 3 of the diazepam-dependent dogs had been infused with the alcoholic solution of flumazenil. The dogs were observed for 5 to 10 minutes after the infusion stopped and while they were in the sling and for at least an hour after they were removed from the sling.

Blood and Tissue Collection

For the time course studies, blood was collected as previously described (34,36). After the dogs had reached the maintenance level of diazepam or flunitrazepam, blood was collected for a 6-hour time course, beginning 1 hour after dosing. For the "trough" plasma levels of the drugs and their metabolites, venous blood samples were obtained 6 hours after the last dose in order to determine the concentrations just before the next dose. "Trough" levels were obtained during the second and eighth week after both groups of dogs reached their maintenance doses as well as during the twelfth week for the flunitrazepam-dependent and fourteenth week for the diazepam-dependent dogs.

At the end of the chronic studies, the maintenance dose of the drug of dependence was administered orally to the dependent dogs. One hour later and while the dogs were under pentobarbital anesthesia, blood samples were taken and the brains were removed, dissected and stored as previously described (28,30).

Plasma Protein Binding

For each flunitrazepam- and diazepam-dependent dog the extent of plasma protein binding of the parent compound and its metabolites (pooled samples for each dog) was determined by equilibrium dialysis. Plasma samples (0.8 ml of each pooled sample) were dialyzed in duplicate at 37°C for 20 h. Multicavity microdialysis cells (Bolab), Spectra/por membrane (mol.wt. cut off 12,000–14,000) (Spectrum Medical) and an isotonic phosphate buffer pH 7.4 were used. Concentrations of flunitrazepam, diazepam and their metabolites were determined in dialysate and in plasma by HPLC. Free fractions were calculated as the ratio of free (dialysate) and total (plasma) drug concentrations.

Drug Analysis

Brain tissue was homogenized with cold normal saline (1:4). Duplicate 250 μl aliquots of homogenate were adsorbed on a buffered (pH 9.5) C-18, 3 cc Bond Elut column along with 25 μl of internal standard (oxazepam, 10 μg/ml in methanol for flunitrazepam and flunitrazepam 10 μg/ml for diazepam) added directly to the column. The columns were rinsed with water and 100 μl of methanol and eluted with 600 μl of methanol. The methanol extracts were taken to dryness under nitrogen. The dried residues were dissolved in 25 μl of methanol and 5 μl was injected into a Waters 600 HPLC system with a programmable multiwavelength detector and data module. A reverse phase Supelco column was used. The mobile phase consisted of methanol:acetonitrile:2 mM KH_2PO_4 buffer (pH 3.6) :: 39:1:60 with a flow rate equal to 2.2 ml/min for flunitrazepam and its metabolites and methanol:acetonitrile:2 mM KH_2PO_4 buffer :: 60:1:39 with a flow rate equal to 1.3 ml/min for diazepam and its metabolites. Plasma was extracted, dried and the residue dissolved as previously described (34) using modifications of previously described procedures (26). Plasma extracts (5 μl) were injected into the HPLC using the same column, mobile phase, and column conditions as described above for the brain extracts. The levels for both brain and plasma were measured by comparison of the area ratios (unknown/internal standard) with the area ratios for the known standards. Under the above conditions, the response was linear from 1.25–250 ng injected (plasma) and 2.5 ng to 250 mg injected (brain tissue) for flunitrazepam, diazepam, and their metabolites. The limit of sensitivity was 0.25 ng/μl for plasma and 0.5 ng/μg for the brain. See Table 2 for retention times and recoveries for the above procedures.

TABLE 2

THE RETENTION TIMES IN MINUTES AND PERCENT RECOVERIES OF FLUNITRAZEPAM AND ITS METABOLITES AND DIAZEPAM AND ITS METABOLITES FROM DOG PLASMA AND BRAIN TISSUES

Compound	Rention Time (min)	% Recovery Plasma	% Recovery Brain
FN	10.5	91 ± 8 (8)	98 ± 6 (8)
ACDFN	1.7	80 ± 8 (8)	87 ± 7 (8)
ADFN	2.1	81 ± 6 (8)	88 ± 6 (8)
AFN	2.7	91 ± 8 (8)	89 ± 8 (8)
HFN	6.9	119 ± 17 (8)	45 ± 6 (8)
DFN	7.5	99 ± 8 (8)	67 ± 6 (8)
DZ	8.2	83 ± 6 (9)	70 ± 2 (6)
ND	7.0	73 ± 3 (9)	69 ± 3 (6)
OX	4.0	76 ± 3 (9)	79 ± 3 (6)

Values are expressed as the mean ± S.E. for the number of samples indicated in parentheses.

FN = Flunitrazepam; ACDFN = 7-acetoamidodesmethylflunitrazepam; ADFN = 7-amidodesmethylflunitrazepam; AFN = 7-aminoflunitrazepam; HFN = 3-hydroxyflunitrazepam; DFN = desmethylflunitrazepam; DZ = diazepam; ND = nordiazepam; OX = oxazepam.

Clinical Laboratory Tests

Blood was collected on two occasions for liver profile or the master chem battery of tests which were performed by Smith Kline Bioscience Laboratories, Lexington, KY. Samples were collected from the flunitrazepam-treated dogs one week after treatment was initiated when they had attained a dose of 2 mg/kg/day and 2 months after they had reached the maintenance dose of 7.6 mg/kg/day. Samples were obtained from 2 diazepam-dependent dogs prior to the initiation of diazepam treatment and approximately 2 months after they had attained their maintenance dose of 20 or 36 mg/kg/day. Samples were obtained from the 4 other diazepam-treated dogs about one month after they had reached their maintenance dose of 24 or 36 mg/kg/day and again at 2 months of treatment at the maintenance dose.

RESULTS

Dogs dependent on flunitrazepam or diazepam (Fig. 1), whose doses had been escalated just to the point of weight loss (ap-

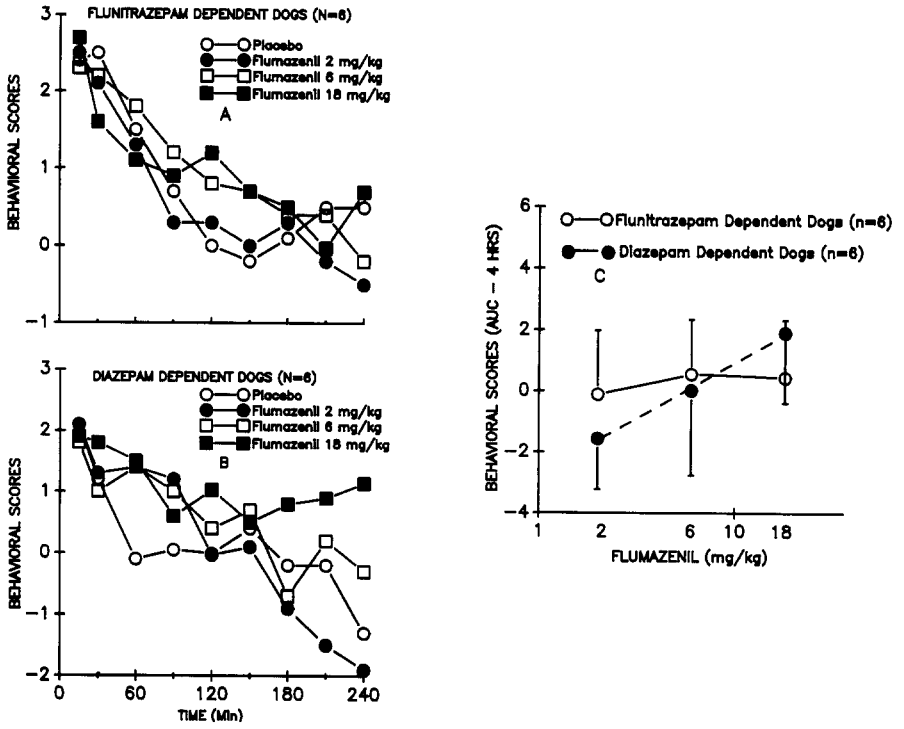

FIG. 2. Time course for behavioral scores produced by graded doses of flumazenil in flunitrazepam-dependent (A) and diazepam-dependent dogs (B). Each point represents the mean for 6 dogs. The time intervals on the X-axis on these and subsequent time-action graphs (Figs. 3 and 4) represent the ending time for each epoch after the administration of flumazenil. The dose-response relationships for the areas under the time-action curves in (A) and (B) are shown in (C). Each point, ±S.E., represents the mean for 6 dogs with the placebo subtracted.

proximately 0.5 kg, range 0.2 to 1.1 kg), neither lost nor gained statistically significant amounts of weight during the stabilization phase. Neither group of dogs showed overt signs of ataxia or sedation.

Flumazenil produced behavioral activation in both flunitrazepam (Fig. 2A)- and diazepam-dependent dogs (Fig. 2B) but this was a dose-related effect for diazepam-dependent dogs only (Fig. 2C). What appears to be a ceiling effect with the 18 mg/kg dose of flumazenil in flunitrazepam-dependent dogs may be related to the fact that one dog went into status epilepticus during the 15–30-min time interval after flumazenil and was anesthetized with pentobarbital. A two-way ANOVA (dogs × doses) of the diazepam behavioral scores (Fig. 2B) expressed as AUC (Fig. 2C) revealed a significant between animals response to flumazenil, $F(5,10)=21.52$, $p<0.001$, a dose-related effect, $F(2,10)=4.48$, $p<0.05$, and a significant regression, $F(1,10)=8.94$, $p<0.025$, with no significant deviation from linearity.

Figure 3 shows the time course for the BPAS scores in flunitrazepam (Fig. 3A)- and diazepam-dependent dogs (Fig. 3B) administered graded doses of flumazenil. Although signs of abstinence emerge within the first few minutes after the administration of flumazenil in dogs dependent on either flunitrazepam or diazepam, the peak BPAS score for each group was seen during the 30- to 60-minute epoch after the administration of flumazenil. The scores obtained in the flunitrazepam-dependent dogs declined steadily after the first hour following flumazenil administration. The BPAS scores for the diazepam-dependent dogs also decreased after the peak effect, but unlike flunitrazepam, a second peak, less intense than the first, emerged 2.5 to 3 hours after flumazenil was administered. The AUC for this second increase in scores (150–240 min, Fig. 3B) had a significant regression with dose, $F(1,10)=6.82$, $p<0.025$. The dose-response curves in Fig. 3C were generated from the four-hour time-action curves in Fig. 3A and B. Although there appears to be a ceiling effect for some signs of abstinence comprising the BPAS scores generated in the flunitrazepam-dependent dogs for the 18 m/kg dose of flumazenil (whole body tremors, hot foot walking and rigid walking), others (clonic seizures, lip licking and twitches and jerks) continued to increase with this dose of flumazenil. A two-way ANOVA (dogs × doses) of the diazepam dose-response curve in Fig. 3C showed a significant between animals response to flumazenil, $F(5,10)=4.34$, $p<0.025$, a dose effect, $F(2,10)=7.98$, $p<0.01$, and a significant regression, $F(1,10)=15.77$, $p<0.005$, with no significant deviation from regression. Similarly, a two-way ANOVA (dogs × doses) of the flunitrazepam dose-response curve in Fig. 3C showed a significant between animals effect, $F(5,10)=7.23$, $p<0.005$, a between doses response to flumazenil, $F(2,10)=5.35$, $p<0.05$, and a significant regression, $F(1,10)=7.80$, $p<0.025$, with no significant deviation from regression. Further, a two-way analysis of variance (dogs × weeks of stabilization) showed no statistically significant between weeks variance for flumazenil-induced BPAS scores for either the flunitrazepam- or diazepam-dependent dogs. Relative potency estimates of these data show that flumazenil was equieffective in precipitating abstinence as measured by the BPAS score in the flunitrazepam- and diazepam-dependent dogs. Using a two-way ANOVA for an incomplete block bioassay, the relative potency and its 95% confidence limits was 0.98 (0.77–

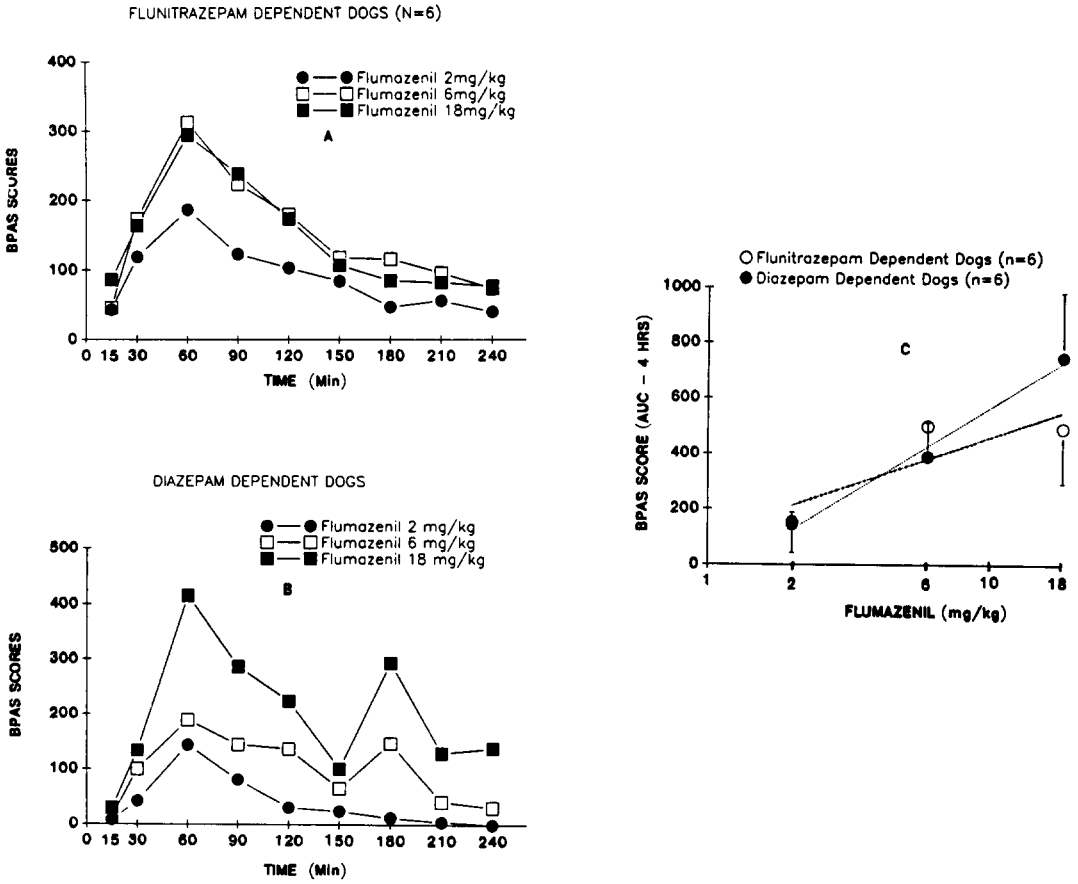

FIG. 3. Time course for BPAS scores produced by graded doses of flumazenil in flunitrazepam-dependent (A) and diazepam-dependent dogs (B). Each point represents the mean for 6 dogs. The dose-response relationships for the areas under the time-action curves in (A) and (B) are shown in (C). Each point represents the mean ± S.E. for 6 dogs with the placebo subtracted. The standard error bars for diazepam (solid circles) are shown in the up direction and those for flunitrazepam (open circles) are in the down direction. The standard error bar for the 6 mg/kg dose of flumazenil in the flunitrazepam-dependent dogs is covered by the symbol for the diazepam-dependent dogs.

1.26). The regression was highly significant ($p<0.001$) and there was no significant between animals variance or deviations from linearity or parallelism. The peak effect for most abstinence signs used in the BPAS score (Fig. 3A and B) occurred during the 30- to 60-minute interval after dosing with flumazenil for both the flunitrazepam-dependent and diazepam-dependent dogs. There were exceptions. The peak effect for twitches and jerks was seen later (90 to 120 minutes) for both drugs of dependence while the peak incidence of rigid walking (not shown) and clonic seizures (Fig. 4A and B) occurred earlier for the flunitrazepam-dependent dogs (30–90 min) and subsided sooner than for the diazepam-dependent dogs (150–180 min).

Although the incidence of clonic seizures was greater for diazepam-dependent dogs than for the flunitrazepam-dependent dogs (Figs. 4B and A; each value is the sum for 6 dogs), a statistically significantly dose-related increase in the average incidence of clonic seizures was observed for the flunitrazepam-dependent dogs only (Fig. 5A and B). The number of dogs having clonic seizures during the 30–60-minute observation period was the same for the flunitrazepam- and diazepam-dependent dogs (Fig. 4C) and the number of dogs having seizures covaried with the number of seizures (Fig. 4A, B, C) during the first 2.5 hours. Seizure activity peaked and then subsided after 2.5 h in flunitrazepam-dependent dogs, whereas the diazepam-dependent dogs exhibited two peaks of seizure activity (Fig. 4A and B). One flunitrazepam- and no diazepam-dependent dog had a flumazenil-precipitated tonic-clonic seizure during this part of the study. The flunitrazepam-dependent dog who had a tonic-clonic seizure with the 18 mg/kg dose of flumazenil went into status epilepticus and was anesthetized with pentobarbital during the 15–30-minute observation period. As indicated by the BPAS scores, some signs comprising these scores showed a peak effect, subsided and then reemerged in the diazepam-dependent (Fig. 4B) but not in the flunitrazepam-dependent dogs (Fig. 4A). These signs included whole body tremors, hot foot walking, rigid walking, tachypnea and clonic seizures.

Overall, all flunitrazepam-dependent (6 of 6) and 5 of 6 diazepam-dependent dogs had clonic seizures either during the crossover study, while rectal temperature was taken after the experiment ended, during a second precipitation with 18 mg/kg of flumazenil, after intravenously administered flumazenil or during the pharmacokinetic studies. Similarly, 6 of 6 flunitrazepam- and 3 of 6 diazepam-dependent dogs had tonic-clonic seizures. When the flunitrazepam- and diazepam-dependent dogs were infused intravenously with flumazenil, a variety of signs of abstinence were seen in addition to clonic and tonic-clonic seizures

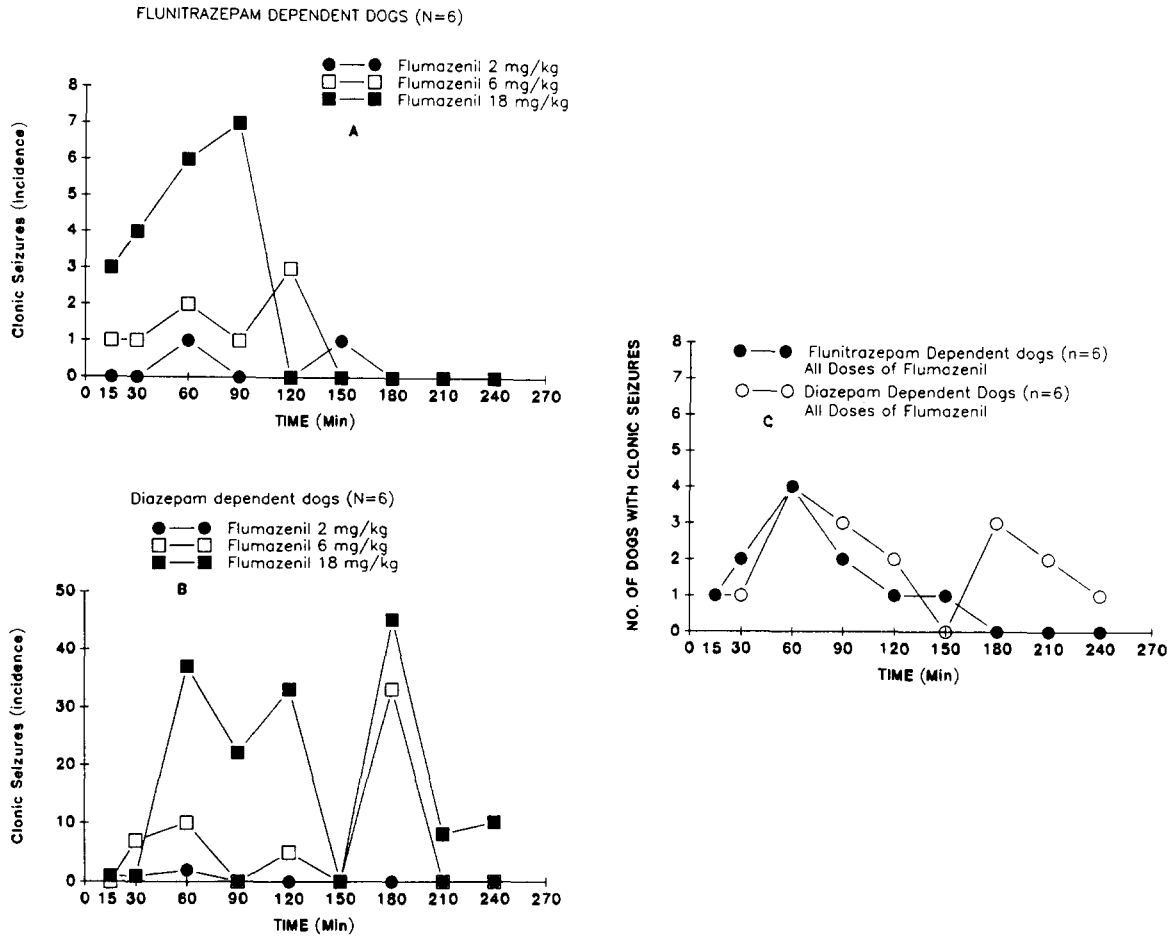

FIG. 4. Time-action curves for clonic seizures produced by graded doses of flumazenil in flunitrazepam (A)- and diazepam-dependent dogs (B). No clonic seizures were observed after a placebo in either the flunitrazepam- or diazepam-dependent dogs, therefore, the placebo is omitted on these graphs. Each value in (A) and (B) represents the number of clonic seizures that occurred in 6 dogs. The number of dogs who had a seizure at each time epoch for three doses of flumazenil combined are shown in (C). If a dog had a seizure on more than one dose of flumazenil during a time period it was counted only 1 time.

including twitches and jerks, tremors, head and limb rigidity, myoclonus, salivation and lip licking. Table 3 summarizes the seizure activity which was observed in these experiments. As can be seen, a lower dose of flumazenil produced seizure activity in the flunitrazepam-dependent dogs than in the diazepam-dependent dogs.

There were signs and behaviors that were not scored in the behavioral or BPAS scores which appeared to be unique, or more prevalent, in the flunitrazepam-dependent than in the diazepam-dependent dogs. Continuous walking around the cage with one front and one back leg on the slanting metal base around the cage was seen in flunitrazepam-dependent dogs but not in diazepam-dependent dogs. Abnormal posturing, which consisted mainly of sitting in a corner of the cage and extending the nose toward the ceiling, was observed in all flunitrazepam-dependent dogs both after a placebo, and after each dose of flumazenil. The diazepam-dependent dogs exhibited this behavior less frequently. It was seen in 2 of 6 diazepam-dependent dogs after a placebo. Retropulsion, which was not observed in either group of dogs after a placebo, was also observed more frequently after flumazenil in the flunitrazepam-dependent than in the diazepam-dependent dogs whereas panting was observed more frequently in the diazepam-dependent than in the flunitrazepam-dependent dogs. In this regard, neither group of dogs had altered body temperatures 4 hours after flumazenil when the experiment ended.

Table 4 presents plasma levels of flunitrazepam, diazepam and their metabolites present 6 hours after the last stabilization dose. Plasma levels of flunitrazepam and diazepam and their metabolites were also obtained for the six-hour interdose period, beginning one hour after receiving the maintenance dose of the drug of dependence, in the absence and presence of flumazenil (Fig. 6A, B, C and D). Peak plasma levels of flunitrazepam were observed between 60 and 90 minutes after dosing. Two major metabolites, 7-aminoflunitrazepam and desmethylflunitrazepam, reached a peak approximately 3 hours after the administration of flunitrazepam and these levels were sustained during the interdose period. Another major metabolite, 7-acetoamidodesmethylflunitrazepam, which is not included on the graphs, showed fluctuating levels during this period. The peak levels of diazepam and oxazepam occurred between 1 and 2 hours after dosing. Diazepam levels were lower than those of oxazepam or nordiazepam and gradually decreased after 2 hours, whereas those of nordiazepam and oxazepam remained stable. Peak plasma levels of flumazenil (6 mg/kg administered orally 1 hour after

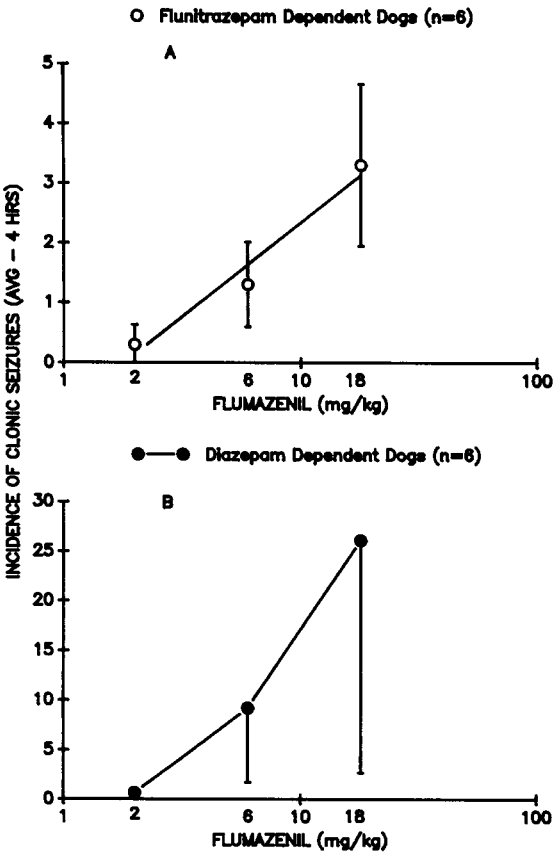

FIG. 5. The dose-response relationships of flumazenil-induced clonic seizures in flunitrazepam- and diazepam-dependent dogs generated from the four-hour time-action curves shown in Fig. 4A and B. Each value, ±S.E., represents the mean number of seizures for each dose of flumazenil for the four-hour observation period (n = 6 dogs) for flunitrazepam-dependent (A) and diazepam-dependent dogs (B).

the drug of dependence) were seen within the first hour after administration in both the flunitrazepam- and the diazepam-dependent dogs (Fig. 6B and D) at a time when many of the signs of abstinence were maximal (Fig. 3A and B; Fig. 4A and B). Although there were no statistically significant differences in the pharmacokinetics of the 6 mg/kg/dose of flumazenil in the flunitrazepam- and diazepam-dependent dogs, there was a tendency for C_{max}, T_{max}, the area under the plasma concentration-time curve and the half-life to be greater in the diazepam-dependent dogs. Pharmacokinetic studies were not conducted for lower and higher doses of flumazenil in the flunitrazepam- and diazepam-dependent dogs; however, in other studies there were no statistically significant differences in the plasma concentration-time curves between 6 and 72 mg/kg of flumazenil in naive dogs and between 2, 6 and 18 mg/kg of flumazenil in nordiazepam-dependent dogs. Table 5 compares brain and plasma bound + free levels, the plasma free fraction and the estimated plasma free concentration of flunitrazepam and its metabolites with those of diazepam and its metabolites 1 hour after receiving the maintenance dose of the drug of dependence. Brain and plasma levels of FN, DFN, HFN, and AFN were not significantly different, whereas brain levels of ADFN and ACDFN were higher than plasma levels. There were no significant differences in brain and plasma levels of diazepam and its metabolites in the diazepam-dependent dogs. Table 5 also shows that the estimated total concentration of bound + free flunitrazepam and its metabolites in plasma are less than the total concentration of bound + free diazepam and its metabolites which is not unexpected taking into consideration the difference in doses. However, it is important to note that plasma protein binding is markedly greater for diazepam and its metabolites than for flunitrazepam and its metabolites. Table 5 further shows that in contrast to the total bound + free concentration, the estimated total free plasma concentration of flunitrazepam and its metabolites tend to be higher than the estimated total free plasma levels of diazepam and its metabolites.

TABLE 3

A COMPARISON OF THE NUMBER OF SEIZURES* INDUCED IN FLUNITRAZEPAM- AND DIAZEPAM-DEPENDENT DOGS BY THE IV INFUSION OF FLUMAZENIL

Flumazenil mg/kg	Vehicle	Clonic Convulsions	Tonic-Clonic Convulsions
	Flunitrazepam		
Avg. 3.8 (range 2.0–6.3)	alcohol	3/6	3/6
	Diazepam		
Avg. 11.6 (5.8–16.6)	alcohol	2/3	1/3
10.0	liposomal suspension	2/2	1/2

*The numerator represents the number of dogs who had seizures and the denominator represents the number of dogs infused.

The only apparent abnormalities indicated by behavioral observation, by examination of postmortem tissue, and by clinical laboratory examination of blood chemistries during the course of these chronic studies was an elevation of serum alkaline phosphatase in the diazepam-dependent dogs and an elevation of serum SGPT and SGOT levels in one of these dogs who died after all experiments were complete (two days after the IV infusion of flumazenil and one hour and 45 minutes after the morning dose of diazepam). This dog had been lethargic for about a week prior to death. Postmortem examination of this dog's tissues showed that the liver was yellowish and enlarged. Normal blood chemistries were found in the flunitrazepam-dependent dogs except for 1 dog who had elevated serum levels of alkaline phosphatase, SGOT and SGPT.

DISCUSSION

The chronic administration of flunitrazepam (7.6 mg/kg/day) produces a degree of physical dependence in the dog quantitatively similar to that produced by diazepam (20 to 36 mg/kg/day) as revealed by the flumazenil-precipitated abstinence syndrome and as measured by the BPAS score. Both binding and pharmacologic studies indicate that flunitrazepam is from 2 to 10 times as potent as diazepam (4, 23, 24, 32). The doses selected in the present study were the highest tolerated without significant anorexia or weight loss and resulted in doses of diazepam that were 2.6 to 4.7 times greater than the dose of flunitrazepam.

Plasma levels of flunitrazepam observed after its chronic administration in the dog are in fairly good agreement with those reported after its prolonged administration in man (3,37). The half-life of flunitrazepam in man (~3 h) is longer, however, than observed in the present studies in the dog (~1.7 h). This agrees

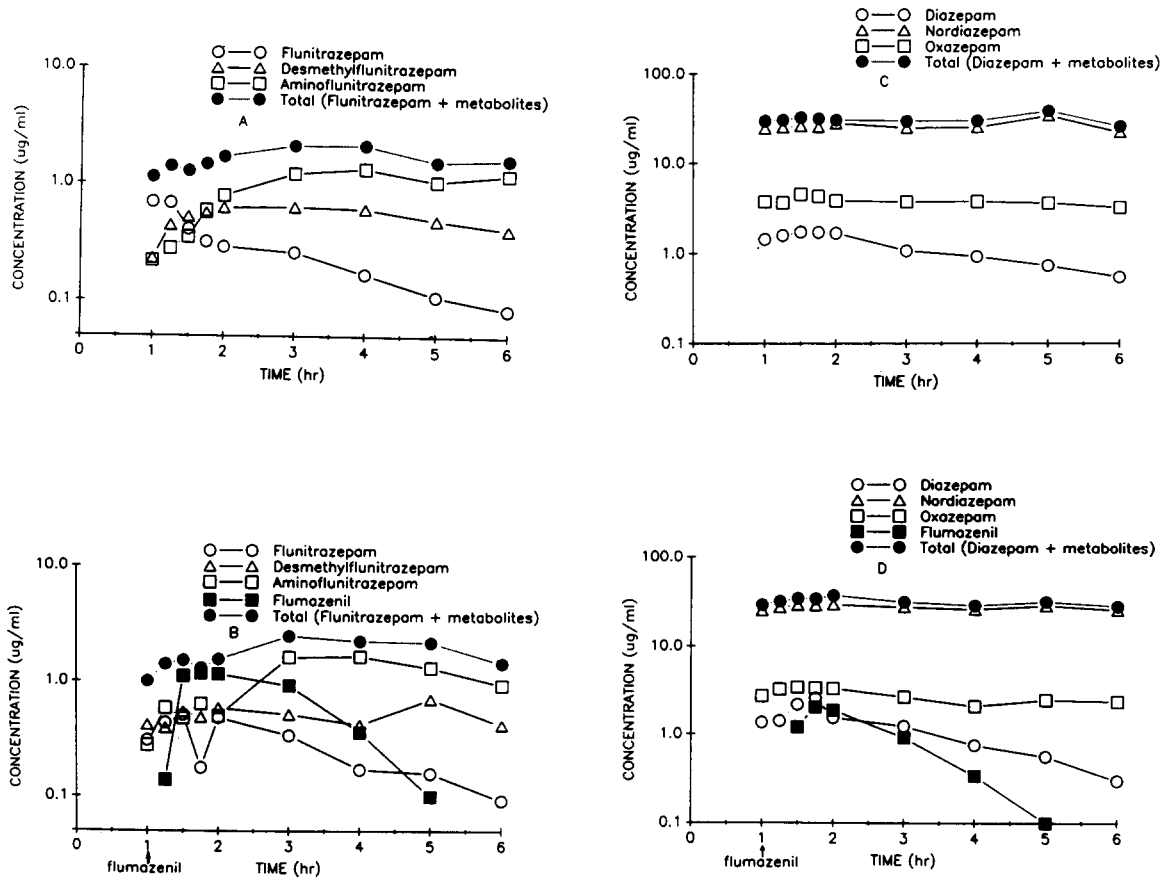

FIG. 6. Stabilization plasma levels of flunitrazepam and its metabolites and diazepam and its metabolites in the absence (A and C) and presence of flumazenil, 6 mg/kg, (B and D) during the 6-hour interdose period. The arrow (B and D) indicates when flumazenil was administered (1 hour after the oral administration of the drug of dependence). Each value represents the mean for 6 dogs.

TABLE 4

MEAN PLASMA LEVELS (μg/ml) ± SEM OF FLUNITRAZEPAM AND DIAZEPAM AND THEIR METABOLITES IN FLUNITRAZEPAM- AND DIAZEPAM-DEPENDENT DOGS

Drug Detected	Time After Stabilization				
	2 Weeks	8 Weeks	10 Weeks	12 Weeks	14 Weeks
			Flunitrazepam		
FN	0.381 ± 0.229	0.427 ± 0.225		0.415 ± 0.212	
DFN	0.668 ± 0.313	0.452 ± 0.264		0.859 ± 0.112	
HFN	0.416 ± 0.233	0		0	
AFN	0.181 ± 0.109	0.296 ± 0.159		0.079 ± 0.032	
ADFN	0.039 ± 0.039	0		0	
ACDFN	1.226 ± 0.274	0.083 ± 0.083		0.221 ± 0.127	
			Diazepam		
DZ	0.161 ± 0.091	0.917 ± 0.119	0.746 ± 0.194		0.673 ± 0.579
ND	20.834 ± 0.857	18.910 ± 2.69	27.530 ± 3.46		23.162 ± 3.399
OX	1.063 ± 0.352	3.548 ± 0.849	3.325 ± 0.999		2.951 ± 0.689

FN = Flunitrazepam; DFN = desmethylflunitrazepam; HFN = 3-hydroxyflunitrazepam; AFN = 7-aminoflunitrazepam; ADFN = 7-amidodesmethylflunitrazepam; ACDFN = 7-acetoamidodesmethylflunitrazepam; DZ = diazepam; ND = nordiazepam; OX = oxazepam.

TABLE 5

A COMPARISON OF BRAIN AND PLASMA LEVELS OF FLUNITRAZEPAM AND DIAZEPAM AND THEIR METABOLITES ONE HOUR AFTER THE ORAL ADMINISTRATION OF THE MORNING STABILIZATION DOSE OF THE DRUG OF DEPENDENCE AND WHILE THE DOGS WERE UNDER PENTOBARBITAL ANESTHESIA

Drug	Brain (μg/g)	Plasma Bound + Free (μg/ml)	Plasma Free Fraction (%)	Estimated Plasma Free Concentration (μg/ml)
		Flunitrazepam-Dependent Dogs		
FN	0.38 ± 0.13	0.17 ± 0.09	41.1 ± 6.7	0.07
DFN	1.06 ± 0.12	0.83 ± 0.14	44.9 ± 11.1	0.37
HFN	0.53 ± 0.08	0.64 ± 0.16	28.3 ± 4.3	0.18
AFN	1.09 ± 0.13	1.39 ± 0.37	38.1 ± 1.2	0.53
ADFN	0.31 ± 0.05$^{0.01}$	0.06 ± 0.04	N. D.	
ACDFN	1.55 ± 0.11$^{0.05}$	1.16 ± 0.21	51.9 ± 10.4	0.60
Total		4.25		1.75
		Diazepam-Dependent Dogs		
DZ	1.46 ± 0.24	1.29 ± 0.26	12.0 ± 1.8	0.17
OX	3.23 ± 0.66	2.19 ± 0.37	8.1 ± 1.0	0.26
ND	19.95 ± 3.72	13.87 ± 1.86	3.7 ± 0.8	0.74
Total		17.35		1.17

Superscripts are p values of differences in brain and plasma bound + free levels.
Values represent the mean ± S.E. for 6 dogs.

with the half life of 1.3 hours found by others in the dog (2). There are other differences in the way chronically dosed humans and dogs metabolize flunitrazepam. AFN and ADFN plasma levels were markedly higher in man than in the dog whereas another metabolite, ACDFN, which was present in significant levels in the dog, has not been reported in man. Thus the metabolism of flunitrazepam, which is nearly complete in the dependent dog, is more complicated than that of diazepam. Although metabolites play a major role in the development of dependence in dogs treated chronically with diazepam (18), it is not known to what extent flunitrazepam's metabolites contribute to the dependence produced by chronically administered flunitrazepam. The total plasma levels of flunitrazepam and its metabolites were lower than the total plasma levels of diazepam and its metabolites in the diazepam-dependent dogs. This was not unexpected since the chronic dose of flunitrazepam was lower than the chronic dose of diazepam. However, if it is assumed that the metabolites of flunitrazepam do not contribute to flunitrazepam's dependence-producing process, then plasma levels of diazepam and its active metabolites are more than 100 times higher than the plasma levels of flunitrazepam. The flunitrazepam-dependent dog, however, had a precipitated abstinence syndrome that was as intense as that seen in the diazepam-dependent dog. The following findings offer an explanation for these observations: 1) The HPLC procedures used in these studies do not distinguish between the bound and free fractions of benzodiazepines present in plasma and tissue. Microdialysis experiments have shown that the concentration of the free rather than the total (bound + free) plasma levels of diazepam, nordiazepam and their metabolites approximates their concentration in the brain extracellular fluid compartment (6,33). Although microdialysis studies were not conducted in the flunitrazepam-dependent dogs, the levels of flunitrazepam and its metabolites in the brain were found to equal or exceed the levels (bound + free) in plasma. For these reasons, plasma protein binding studies were conducted and showed that diazepam and its metabolites are bound to plasma proteins to a markedly greater degree than flunitrazepam and its metabolites. Thus, in contrast to the total (bound + free) concentration, the concentration of free flunitrazepam and its metabolites exceeded the concentration of diazepam and its metabolites in plasma. 2) Flunitrazepam concentrations were elevated early in the interdosing period, following which AFN concentrations increased when flunitrazepam concentrations were falling. Both AFN and DFN plasma levels as well as the total plasma levels of flunitrazepam and its metabolites were sustained throughout the interdosing period. Whereas flunitrazepam represented an appreciable portion of the free drugs found in the plasma, the metabolites were present in higher concentrations than flunitrazepam. It is conceivable that a parent drug such as flunitrazepam with a short half-life could act in concert with an active metabolite to sustain activity through the interdosing period and play a role in flunitrazepam's dependence-producing properties. The issue of sustained concentration levels may be an important variable in the production of dependence. In diazepam- and nordiazepam-dependent dogs, nordiazepam and oxazepam levels are sustained through the interdose interval and for many hours longer (17,22). Signs of withdrawal abstinence do not appear until plasma levels of nordiazepam begin to fall (17). It has also been shown that the sustained slow release of small amounts of diazepam from silastic capsules produces a degree of physical dependence in the rat (a species which metabolizes less diazepam to nordiazepam than man or the dog) characterized by flumazenil precipitated tonic-clonic seizures (31,38). Thus both diazepam and its metabolites probably play a role in its dependence-producing properties. Both AFN and DFN have been shown to have agonistic activity (11,23); however, it should be emphasized that the pharmacology of flunitrazepam's metabolites has not been systematically investigated and their contribution to the dependence process is unknown.

Although flumazenil itself can decrease behavioral and BPAS scores in the naive dog (22,30), it produced behavioral activation and increased BPAS scores in the flunitrazepam- and diazepam-dependent dogs. Further, no clonic or tonic-clonic seizures have ever been observed in flumazenil-treated naive dogs or in benzodiazepine-dependent dogs administered a lactose placebo (19, 22, 30). These findings indicate that the precipitated absti-

nence syndromes observed in the present study cannot be attributed to an agonistic or inverse agonistic action of flumazenil or to withdrawal effects of the drug of dependence during the interdose interval of the observation period.

Several factors may play a role in the intensity of the precipitated abstinence syndrome: 1) It has been reported that tolerance develops to flumazenil's ability to precipitate abstinence in diazepam-dependent baboons and rhesus monkeys when it is administered every third day (10,13). No apparent tolerance developed to this effect of flumazenil in the present studies in the dog where the interval between doses was longer (1 week). The evidence for this is that there was no statistically significant between weeks variance when the BPAS scores obtained in the diazepam- and flunitrazepam-dependent dogs were analyzed using a two-way ANOVA. 2) Another factor that plays a role in the intensity of the benzodiazepine-precipitated abstinence syndrome is the rate of the development of dependence. Several investigators have shown that the intensity of diazepam-precipitated abstinence increases as the dose and/or duration of chronic treatment with diazepam increases in baboons, rats and dogs (10, 15, 16, 38). The chronic exposure to diazepam in these studies did not exceed 35 days. In the present crossover studies where a stabilization dose of diazepam or flunitrazepam was administered chronically in higher doses and for a longer period (4.1 to 7.5 weeks), no statistically significant between weeks variance was found. It should be further pointed out that the total exposure time to the drug of dependence was even longer (7.4 to 10.5 weeks). In this regard, it has been shown that in cats treated chronically with diazepam and flurazepam for a period of 70 days, the flumazenil-precipitated abstinence syndrome is near maximal after 7 days of exposure (27). Similarly, only a modest increase in flumazenil-induced NPAS scores was observed across 18 weeks of chronic treatment with nordiazepam in the dog (29).

The flumazenil-precipitated abstinence syndromes in the flunitrazepam- and diazepam-dependent dogs were quantitatively similar although qualitative differences were observed: 1) Differences were observed in the time course curves for the BPAS scores and the signs comprising these scores. The signs of abstinence as measured by the BPAS score are most intense in both the flunitrazepam- and diazepam-dependent dogs during the 30–60-minute observation period following the administration of oral flumazenil. This peak effect corresponds to the time when flumazenil plasma levels were the highest (1–2 h) after the oral administration of 6 mg/kg in both the flunitrazepam- and diazepam-dependent dogs. Whereas recovery continues after the peak abstinence effect for the remainder of the experiment in the flunitrazepam-dependent dogs, the signs reemerge in the diazepam-dependent dogs and reach a second, but lesser peak during the 150–180-minute observation epoch. Reasons for the reemergence of abstinence in the diazepam-dependent dogs are probably complex and involve several factors: (a) The second abstinence peak occurred at a time when both the plasma flumazenil (~17% of maximum) and diazepam (~29% of maximum levels) were declining but while nordiazepam levels were maintained (~ 90% of peak levels). In this regard, it has been found that in humans, the EEG profiles, conditioned reaction time and clinical ratings for anxiety and sedation all showed significant changes after IV diazepam which were different in the presence and absence of nordiazepam. It has been postulated that nordiazepam can antagonize some of the effects of diazepam at the receptor level (7). Thus, if nordiazepam can act centrally to antagonize some of diazepam's agonistic effects, the increased ratio of nordiazepam/diazepam in the brain extracellular fluid compartment could conceivably contribute to the reemergence of abstinence effects. (b) There was a tendency for the AUC and the t½ for plasma flumazenil to be greater in diazepam- than in flunitrazepam-dependent dogs. Further, it has been shown that the pharmacokinetics of flumazenil are different in dogs dependent on different benzodiazepines which may be due to differences in protein binding [(35,36), Wala et al., in preparation]. Hence the drug of dependence and/or its metabolites may alter the levels of free flumazenil at receptor sites. 2) Differences were also observed in several unscored behaviors and signs that were either unique or more prevalent in the flunitrazepam- than in the diazepam-dependent dogs. These were abnormal posturing, walking on the side of the cage and retropulsion. Although benzodiazepines are generally thought to exert their pharmacologic effects through a specific high affinity benzodiazepine binding site in the CNS which is modulated by gamma-aminobutyric acid and chloride (14,25), studies of the dependence-producing properties of different benzodiazepines and/or their metabolites indicate that these precipitated abstinence syndromes are qualitatively different, suggesting that they may have different mechanisms and sites of action (22).

The clinical evidence concerning the physical dependence-producing properties of flunitrazepam in man appears to be limited. Seven cases have been reported (8) in which patients who were using flunitrazepam alone (1 case) or with other benzodiazepines (6 cases) had tonic-clonic seizures following withdrawal. Flunitrazepam is prescribed mostly for nighttime sedation and hypnosis and is taken in single doses of 0.5–2.0 mg whereas diazepam is frequently taken chronically several times daily in doses of 4–40 mg per day. These differences in patterns of use may contribute to the difference in reported incidence of cases of dependence. The possibility that flunitrazepam may have a greater dependence-producing capacity than diazepam in the dog than in man must be considered, particularly in view of the differences in metabolism between the two species.

In conclusion, chronically administered flunitrazepam and diazepam in doses which induced a transient loss of body weight produced physical dependence in the dog. Although the precipitated abstinence syndromes observed in the flunitrazepam- and diazepam-dependent dogs show more similarities to each other than to those precipitated in any other benzodiazepine studied (22), they do show differences. These differences may be related to differences in the pharmacology of their metabolites. It is concluded that the flunitrazepam- and diazepam-dependent dogs did not differ in the intensity of precipitated abstinence (relative potency = 1) and hence the differences in the syndromes probably cannot be attributed to the level of physical dependence.

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