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Dependence-Producing Properties of Alprazolam in the Dog

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SLOAN, J. W., W. R. MARTIN AND E. P. WALA. *Dependence-producing properties of alprazolam in the dog.* PHARMACOL BIOCHEM BEHAV **35**(3) 651–657, 1990.—Alprazolam (48 mg/kg/day) administered orally to dogs 4 times a day in equally divided doses produced physical dependence. This dependence was revealed by a precipitated abstinence syndrome which occurred after either oral administration of flumazenil (6, 18 and 36 mg/kg) or intravenous administration of a liposomal suspension of flumazenil. Flumazenil alone (18, 36 and 72 mg/kg) produced no significant signs of precipitated abstinence in naive dogs. This precipitated abstinence syndrome in alprazolam-dependent dogs was characterized by both clonic and tonic-clonic seizures. Other signs of precipitated abstinence which comprise the NPAS score were less intense in the alprazolam-dependent than in diazepam-dependent dogs. Alprazolam is extensively metabolized in the dog and does not accumulate whereas its predominant metabolite, alpha hydroxyalprazolam, does accumulate. The data suggest that alpha hydroxyalprazolam plays a role in the dependence-producing properties of alprazolam in the dog as revealed by the precipitated abstinence syndrome.

Alprazolam dependence Flumazenil precipitated abstinence in dogs RO15-1788 Alprazolam
Flumazenil plasma levels Alprazolam plasma levels Alprazolam brain levels

ALPRAZOLAM is a widely employed benzodiazepine which is used for the treatment of anxiety and as a sedative and antidepressant. Animal studies indicate that it also has anticonvulsant as well as muscle relaxant properties (13, 24, 27, 28). Clinical data suggest that it is approximately ten times more potent than diazepam (1, 5, 22) and binding data obtained in rats indicate that its dissociation constant is several times smaller than diazepam's (15, 21, 30, 31). It has recently been identified (6) as having a relatively large number of emergency room mentions and about 5 percent of these mentions are related to dependence. The metabolism, distribution and excretion of alprazolam is complex in man. The major metabolites, however, are alpha-hydroxyalprazolam, 4-hydroxyalprazolam and desmethyl-alprazolam (8–10). The present study was conducted to determine the ability of alprazolam to produce physical dependence in the dog as measured by the intensity of the precipitated abstinence syndrome and to relate these findings to plasma and brain levels of alprazolam and its metabolites (alpha-hydroxyalprazolam and 4-hydroxyalprazolam). These studies, however, do not address the issue of withdrawal abstinence.

METHOD

Subjects

The methods employed in these studies have been previously described (19,20). Six female drug naive beagle-type dogs weighing approximately 10 kg were used. The dogs were dosed orally with gradually increasing amounts of alprazolam administered in No. 4 gelatin capsules 4 times daily (0700, 1300, 1900 and 2400 hr) and in equally divided doses [beginning with 0.2 mg/kg/day (2 dogs) or 0.8 mg/kg/day (4 dogs)] until a dose level of 48 mg/kg/day was attained after 18 to 26 days. Attempts were made to increase the dose to the point of weight loss (19,20). No weight loss occurred during the dose "run-up"; in fact, there was a tendency for the dogs to gain weight. In this regard, a "trial precipitation" was conducted when the animals attained a stabilization dose level of 28 mg/kg/day. At this dose level, few signs of abstinence and no seizures were observed. For this reason, the dose was increased to 48 mg/kg/day, again with no weight loss. During the course of the precipitation experiment the dogs neither gained nor lost weight although there was a slight but non-significant tendency to lose weight. The dogs received the maintenance dose of 48 mg/kg/day (12 mg/kg q.i.d.) for at least a week before precipitation studies were initiated. A similar group of 6 female drug naive beagle-type dogs that were housed and acclimated as above were used to assess the effects of flumazenil alone.

Precipitated Abstinence

Dogs were brought to the observation cages at 0700 the day before the experiment and were food but not water deprived at

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TABLE 1

CHRONOLOGY OF THE ALPRAZOLAM EXPERIMENTS (SEE THE METHOD SECTION FOR DETAILS)

I.	ACCLIMATION: The dogs were kept in the animal care facility for 1.5–3 months prior to initiation of the chronic studies in order to verify their health and to allow them to adjust to their environment.
II.	ALPRAZOLAM DOSE ESCALATION: The dose was gradually increased over a period of 18 to 26 days until a dose of 12 mg/kg q.i.d. was attained.
III.	STABILIZATION PERIOD: The dogs were held at the maintenance dose of 12 mg/kg q.i.d. for about 1 week prior to the initiation of flumazenil-precipitated abstinence.
IV.	FLUMAZENIL-PRECIPITATED ABSTINENCE, CROSSOVER EXPERIMENTS: These experiments were conducted weekly during the first 3 weeks following stabilization.
V.	PHARMACOKINETIC TIME-COURSE AND TROUGH LEVELS OF ALPRAZOLAM: These studies were conducted during the 2nd week after the stabilization period. Samples were collected twice during this week for trough level.
VI.	LACTOSE PLACEBO ADMINISTRATION: Each dog received a lactose placebo during the 4th week after the stabilization period.
VII.	FLUMAZENIL PHARMACOKINETIC TIME-COURSE: These studies were conducted during the 4th week after the initial stabilization period. [These studies are reported herein for seizure activity only. Pharmacokinetic results are in preparation (Wala *et al.*).]
VIII.	PRECIPITATED ABSTINENCE EMPLOYING INTRAVENOUSLY ADMINISTERED FLUMAZENIL: These experiments were conducted during the 5th and 6th weeks after the initial stabilization period while the dogs were still in the sling.
IX.	PLASMA LEVELS OF FLUMAZENIL AND ALPRAZOLAM AND ITS METABOLITES: Intravenous blood was collected approximately 5 minutes after the injection of flumazenil in step VIII above.
X.	BRAIN AND PLASMA LEVELS OF ALPRAZOLAM AND ITS METABOLITES 1 HOUR AFTER THE MAINTENANCE DOSE OF 12 mg/kg OF ALPRAZOLAM: These experiments were conducted at the end of the study during the 5th and 6th weeks after the initial stabilization period.

1900. The maintenance dose of 12 mg/kg of alprazolam was administered to the alprazolam-dependent dogs at the regular time of 0700 on the day of the experiment. The naive control dogs were not dosed at 0700. Rectal temperature and body weight were determined just prior to the oral administration of flumazenil or a lactose placebo at 0800 and at the end of the experiment 4 hours later. Precipitation studies were conducted at weekly intervals on a blind basis using a replicate block 3×3 Latin square design. Two observers (W.R.M. and J.W.S) observed the same two dogs at every precipitation study and tabulated the occurrence of a number of signs and behaviors on an observation sheet. Three doses of flumazenil (6, 18 and 36 mg/kg) were used to precipitate abstinence in the alprazolam-dependent dogs during the first three weeks. The fourth week, a lactose placebo was administered. The lactose placebo was not included in the Latin square design since previous studies have shown that the NPAS scores for the placebo did not differ significantly in benzodiazepine-dependent dogs (e.g., flunitrazepam) where the observers were blind to the treatment compared to groups where the observers knew the dogs received a placebo. Since these experiments are expensive it was decided to use the 3×3 replicate Latin square design rather than the 4×4 replicate Latin square design (see the Discussion section). The naive control dogs also received flumazenil and a lactose placebo according to the same design except the doses of flumazenil were 18, 36 and 72 mg/kg. Both groups of dogs were observed for a number of signs and these were recorded for the following intervals: 0.25, 0.5, 1, 2, 3, and 4 hours after the administration of flumazenil. A modified Nordiazepam Precipitated Abstinence Scale (NPAS) comprised of four signs of abstinence was used to calculate abstinence scores (20). These signs and their assigned weights are: gross tremor (3); twitches and jerks (1); hot-foot walking (2); and respiratory rate (1). The weighting values were assigned such that each sign made an approximately equal contribution to the scale score. The area under the time-action curve for each sign, for each dog and for each dose of flumazenil and the lactose placebo was determined by the trapezoidal rule. The NPAS score was then calculated by adding the weighted areas obtained for each of these four signs. All values are presented with the lactose placebo value subtracted. 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 residual or deviation from linearity variance. The incidence of clonic and tonic-clonic convulsions was statistically evaluated using a chi-square analysis.

Approximately 1 week after the completion of the crossover experiments, each dog was transferred to an observation room and placed in a dog sling approximately 2 hours after the morning stabilization dose of alprazolam. While in the sling a liposomal suspension of flumazenil (5%, Hoffmann-La Roche) was injected IV into a leg vein over a period of 29.6 ± 6.6 seconds (until either a clonic or tonic-clonic convulsion or extreme nuchal or limb rigidity was induced). The dogs were observed for 5 to 10 minutes while they were in the sling and for at least an hour after they were removed from the sling. The 6 naive dogs also received an IV suspension of flumazenil, ~10 mg/kg, about 1 week after completion of the crossover studies (see Table 1 for the chronology of the studies reported herein).

Blood and Tissue Collection

Acute studies. Alprazolam (12 mg/kg) was administered to three benzodiazepine naive dogs approximately 5–6 weeks after they had completed the flumazenil crossover studies. Blood samples were taken and the brains removed 1 hour after the alprazolam dose and while the dogs were under pentobarbital anesthesia. Plasma was obtained and frozen at -20 degrees centigrade as previously described (36). Brains were removed and

dissected as previously described (33) except the animals were not subjected to exsanguination. The brains were chilled and dissected into the following areas: frontal, parietal, temporal, occipital and pyriform cortex, cingulum, caudate, fornix and hippocampus, remaining striatum, cerebellum, medulla, pons, mesencephalon, thalamus and hypothalamus. The tissue was then weighed, sealed in plastic bags and rapidly frozen in dry ice-acetone prior to storage at −70 degrees centigrade until they were analyzed.

Chronic studies. For the time-course studies, blood was collected as previously described (36). After the dogs had reached the stabilization dose of alprazolam, blood was collected during a 6-hour time-course, beginning 30 minutes after dosing. For the trough plasma levels of alprazolam and its metabolites, venous blood samples were obtained 6 hours after the last dose in order to determine the concentrations just before the next stabilization dose.

Venous blood samples were collected after the crossover studies approximately 5 minutes after the end of the IV infusion of the liposomal suspension of flumazenil in the alprazolam-dependent dogs while the dogs were still in the sling.

At the end of the chronic study, blood samples were taken and the brains removed from the 6 alprazolam-dependent dogs 1 hour after the last maintenance dose of alprazolam and while they were under pentobarbital anesthesia. The brains were dissected, weighed, packaged and stored as described previously for the acute studies.

Drug Analyses

Plasma drug levels. Plasma levels of alprazolam and its metabolites were estimated by HPLC according to modifications (36) of a previously described procedure (26). Nordiazepam was used as the internal standard. The limit of sensitivity was 1.25 ng injected or 0.15 μg/ml of the original plasma. Percent recoveries ± standard errors were 84.0 ± 3.1, 96.2 ± 7.5 and 96.1 ± 6.3 for 4-hydroxyalprazolam, alpha-hydroxyalprazolam and alprazolam, respectively.

Brain drug levels. Bond Elut columns (C18, 3 cc) were used to extract alprazolam and its metabolites from NaCl homogenates of brain tissues. The drugs were then eluted from the columns with methanol and the drug concentrations were estimated by HPLC using nordiazepam as the internal standard according to modifications of previously described procedures [(36) and Wala *et al.*, in preparation]. The limit of sensitivity for the method was 0.25 μg/gram of tissue. Percent recoveries and their standard errors were: alprazolam, 86.1 ± 6.3; 4-hydroxyalprazolam, 84.0 ± 3.1 and alpha-hydroxyalprazolam, 86.2 ± 7.5. The concentrations of alprazolam and its metabolites were estimated in 10 brain areas. A two-way analysis of variance revealed that there was no significant difference between the brain areas for either alprazolam, 4-hydroxyalprazolam or alpha-hydroxyalprazolam. Therefore, whole brain concentration for each metabolite was calculated from the area concentrations as the weighted mean accounting for differences in the mass of different areas for each dog. The values are presented as the mean of all dogs for each group (naive, n = 3, and alprazolam dependent, n = 6). Pentobarbital, which was present in plasma and tissues of the dogs when they were sacrificed, did not interfere with the HPLC estimation of alprazolam and its metabolites.

Statistical analysis of plasma and brain levels of drugs. The evaluation of pharmacokinetic parameters was accomplished by model-independent analysis (14). The elimination rate constants were estimated by linear regression analysis of the terminal part of the log plasma concentration-time curves. The half-lives were then obtained from the corresponding rate constants. The data were further analyzed by a two-way analysis of variance (dogs × brain area drug levels or dogs × weeks) and by unpaired *t*-tests.

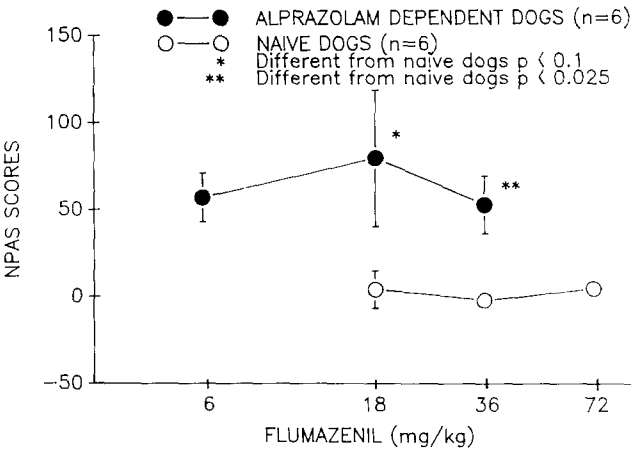


FIG. 1. Dose-response relationship of NPAS scores in alprazolam-dependent and naive dogs receiving graded doses of flumazenil. Values represent the mean ± S.E.M. The S.E.M. bars for the 36 and 72 mg/kg doses do not show above and below the symbols for the naive dogs.

RESULTS

Although very large doses of alprazolam were administered daily no weight loss was observed nor were there any overt signs of sedation or ataxia. Preliminary precipitation studies indicated larger doses of flumazenil would be required to precipitate abstinence in the alprazolam-dependent dogs than were required to precipitate abstinence in diazepam-dependent dogs (2, 6, and 18 mg/kg) (20). The NPAS scores (less lactose placebo) are illustrated in Fig. 1 where alprazolam NPAS area scores are compared with scores obtained in flumazenil-treated naive dogs. A two-way analysis of variance revealed that there was no significant between dogs or between doses effect of flumazenil in naive dogs. The naive dogs did not show any significant increase in the NPAS area score above placebo treatment. Further, the NPAS scores observed for the alprazolam-dependent dogs were statistically significantly higher than the lactose placebo ($p>0.025$) for both the 6 and 36 mg/kg doses of flumazenil and marginally significantly higher for the 18 mg/kg dose of flumazenil ($p>0.1$). As can be seen, the alprazolam NPAS scores are higher than those seen in the naive dogs. A two-way analysis of variance for the scores obtained for the alprazolam-dependent dogs revealed that there was a significant between dogs difference in variance, $F(5,10)=3.38$, $p<0.05$, but no significant between doses effect. There was, however a significant deviation from linear regression, $F(1,10)=5.27$, $p<0.05$ (Fig. 1). There was no statistically significant between weeks difference in variance for either the alprazolam-dependent or the naive dogs. It should be mentioned that one alprazolam-dependent dog exhibited a severe and prolonged precipitated tonic-clonic convulsion following the 18 and 36 mg/kg doses of flumazenil and was judged to be in status epilepticus. Previous experiences had indicated that if this condition was not treated the dog was likely to die. For this reason the dog was anesthetized with sodium pentobarbital before all the observations were made. Therefore, the NPAS area scores are underestimated for the 18 and 36 mg/kg doses of flumazenil.

Table 2 shows the incidence of flumazenil-precipitated clonic and tonic-clonic seizures in the alprazolam-dependent dogs. There was a statistically significant increase in the incidence of tonic-clonic seizures with increasing doses of flumazenil. No dog that

TABLE 2

THE INCIDENCE OF FLUMAZENIL-PRECIPITATED CONVULSIONS IN ALPRAZOLAM-DEPENDENT DOGS AND NAIVE DOGS

Flumazenil Dose (mg/kg); Route of Administration	Clonic Convulsions	Tonic-Clonic Convulsions
6 mg/kg, PO*	1/6†	0/6
18 mg/kg, PO	5/6#	3/6¶
36 mg/kg, PO	5/6#	5/6#
2–7. 5 mg/kg, IV‡	4/6¶	5/6#
6 mg/kg, PO§	4/6¶	1/6

*PO challenges conducted using Latin square design (see text for further details).

†Values represent the number of dogs exhibiting convulsions/number of dogs tested.

‡IV administration (flumazenil in liposomal suspension) to dogs in restraining slings (see text for further details).

§Oral administration of flumazenil, 1 hour after the maintenance dose of 12 mg/kg of alprazolam to alprazolam-dependent dogs in pharmacokinetic time-course study (Wala *et al.*, in preparation).

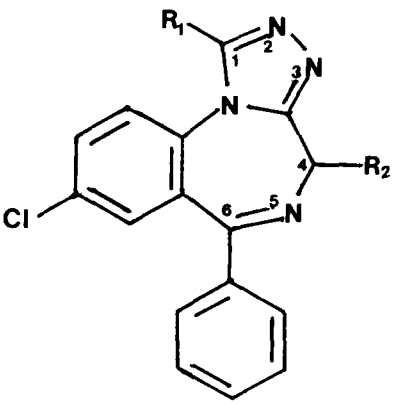
Flumazenil administered orally (18, 36 and 72 mg/kg to naive dogs, n = 6) did not elicit clonic or tonic-clonic convulsions in any dogs. Further, no convulsions were observed in alprazolam-dependent dogs when they were administered a lactose placebo orally (see the Method section).

¶$p<0.05$, the frequency of convulsions is significantly greater in chronic alprazolam-treated dogs relative to naive controls or placebo-treated controls, chi-square for proportions.

#$p<0.01$, the frequency of convulsions is significantly greater in chronic alprazolam-treated dogs relative to naive controls or placebo-treated controls, chi-square for proportions.

had been chronically treated with alprazolam in these studies exhibited either clonic or tonic-clonic convulsions during the course of the precipitation experiment when they were administered a lactose placebo nor did any naive dog have a convulsion during the four-hour observation after graded doses of flumazenil. With the low dose of flumazenil (6 mg/kg) only 1 dog in 6 had a clonic convulsion and none had a tonic-clonic convulsion during the crossover experiments. When the dogs were administered 6 mg of flumazenil in the flumazenil pharmacokinetics time-course study where they were handled repeatedly in order to draw blood, the incidence of convulsions was significantly increased compared with the lactose placebo. Under these circumstances 4 of 6 dogs had clonic convulsions and 1 dog had a tonic-clonic convulsion. With the higher doses of flumazenil (18 and 36 mg/kg) 5 of 6 dogs in the crossover study had clonic convulsions. Three of 6 dogs had tonic-clonic seizures with the 18 mg/kg dose and 5 of 6 dogs had tonic-clonic convulsions with the 36 mg/kg dose. When the dogs were infused intravenously with a liposomal suspension of flumazenil in doses of 2 to 7.5 mg/kg (administered over an average time of 30 seconds) either a clonic or tonic-clonic convulsion was seen in all dogs within 50 seconds of the start of the infusion. Other signs of precipitated abstinence in the IV flumazenil-infused alprazolam-dependent dogs included struggling in the sling, gross body tremors, twitches and jerks, extreme nuchal and limb rigidity and lip licking. Some signs of abstinence in these dogs were obscured by the convulsions and by pentobarbital which was administered to the dog who had tonic-clonic convulsions after the 18 and 36 mg/kg doses of flumazenil.

In two alprazolam-dependent flumazenil-treated dogs, clonic and tonic-clonic seizures were preceded by behaviors which resembled canine delirium. These behaviors were characterized by



$R_1 = CH_3$; $R_2 = H$ (alprazolam)

$R_1 = CH_2OH$; $R_2 = H$ (α-hydroxyalprazolam)

$R_1 = CH_3$; $R_2 = OH$ (4-hydroxyalprazolam)

FIG. 2. Structural formulas of alprazolam and its metabolites in the dog, α-hydroxyalprazolam and 4-hydroxyalprazolam.

TABLE 3

PHARMACOKINETIC PARAMETERS FOR ALPRAZOLAM AND ITS METABOLITES IN ALPRAZOLAM-DEPENDENT DOGS

	C_{max} (μg/ml)	t_{max} (min)	C_{ss} (μg/ml)	K_{el} (min^{-1})	$t_{1/2}$ (min)
Alprazolam	0.91 ±0.17	107.5 ±17.5	0.22 ±0.08	0.06 ±0.02	41.4 ±10.0
α-Hydroxyalprazolam	8.78 ±0.32	120.0 ±19.4	6.13 ±0.58	0.003 ±0.001	228.0 ±33.1
4-Hydroxyalprazolam	1.19 ±0.18	117.5 ±14.5	0.43 ±0.06	0.009 ±0.002	95.1 ±26.0

Values represent the mean ± S.E. (n = 6).

wild running and barking, lunging at something which was not there, wild flinging of the legs, jerking, tremor of the upper lip, shaking, lip licking and nuchal and limb rigidity. These symptoms occurred between 90 and 120 minutes after receiving 36 mg/kg of flumazenil in one dog (3160). This dog then had repeated clonic seizures and interspersed episodes of barking. These behaviors also were seen after the 18 mg/kg dose of flumazenil in another dog (3171) during the same time period. Dog 3171 had clonic convulsions preceding these behaviors (10 minutes and 52 minutes after flumazenil was administered). With the 36 mg/kg dose of flumazenil dog 3171 had repeated clonic convulsions beginning about 20 minutes after it was dosed. What appeared to be canine delirium emerged about 50 minutes after dosing, which was somewhat sooner than seen following the lower dose in this dog or with either dose in dog 3160. Following the delirium dog 3171 had severe tonic-clonic convulsion that progressed to status epilepticus.

TABLE 4

BRAIN AND PLASMA LEVELS (MEAN ± SEM) OF ALPRAZOLAM AND ITS METABOLITES IN DOGS ADMINISTERED SINGLE* AND CHRONIC* DOSES OF ALPRAZOLAM

Treatment	Number of Dogs	Drug Detected	Levels in Plasma (μg/ml)	Levels in Brain (μg/g tissue)	Ratio Brain/Plasma
Single dose (12 mg/kg)	3	4-OHAL	0.18 ± 0.18	0	
		α-OHAL	1.42 ± 0.82	1.08 ± 0.74	0.66 ± 0.14
		AL	1.47 ± 0.53	1.16 ± 0.53	0.66 ± 0.14
Chronic dose (12 mg/kg q.i.d.)	6	4-OHAL	0.31 ± 0.15	0	
		α-OHAL	5.89 ± 0.58†	4.93 ± 0.66†	0.92 ± 0.13
		AL	1.21 ± 0.35	1.41 ± 0.34	1.52 ± 0.36

*Blood samples were obtained 1 hour after a single oral dose of alprazolam (12 mg/kg) was administered to naive dogs or one hour after the morning dose (12 mg/kg) was administered to the alprazolam-dependent dogs.

†Different from single dose, $p<0.025$ by an unpaired t-test.

TABLE 5

COMPARISON OF NPAS SCORES OBTAINED IN NAIVE, ALPRAZOLAM-DEPENDENT AND FLUNITRAZEPAM*-DEPENDENT DOGS AFTER THE ADMINISTRATION OF A LACTOSE PLACEBO

Drug of Dependence	NPAS Score
Naive	57.4 ± 3.7
Flunitrazepam	290.5 ± 144.8
Alprazolam	97.2 ± 12.3†

Values represent the mean ± S.E. for 6 dogs in each group.

*The lactose placebo was administered in a Latin square design on a blind basis to the observers for the flunitrazepam-dependent dogs but not for the naive and alprazolam-dependent dogs.

†Different from the naive dogs, $p<0.05$.

After the intravenous infusion of flumazenil, no naive dog exhibited either a clonic or tonic-clonic convulsion. However, all of these dogs showed mild struggling in the sling immediately after the infusion of flumazenil started and drooping of the head and closure of the eyes such that they appeared to be asleep within 4 minutes. Three of the 6 dogs panted for up to 15 minutes and one dog showed some mild forelimb rigidity. Mild periodic head and body tremors were seen in 4 of the 6 dogs within 5 to 15 minutes. When the dogs were removed from the sling and allowed to walk freely, 2 dogs showed hind limb weakness and ataxia. When returned to the cages after about 15 minutes, all dogs went to sleep.

Alprazolam plasma levels were low (0.25 ± 0.16 μg/ml) 3 hours after the maintenance dose of 12 mg/kg ($n=6$) in dogs stabilized for 5 weeks. The only metabolites detected were 4-hydroxyalprazolam (0.47 ± 0.13 μg/ml) and alpha-hydroxyalprazolam (5.79 ± 1.17 μg/ml). Figure 2 shows the structure of alprazolam and the location of the 4 and alpha positions. These metabolites were found in all 6 dogs. The trough levels of alprazolam (6 hours after the maintenance dose) were too low for accurate determination whereas the plasma levels of 4-hydroxyalprazolam were 0.21 ± 0.07 μg/ml and the levels of the major metabolite, alpha-hydroxyalprazolam, were 4.30 ± 0.41 μg/ml.

Abstinence was also precipitated in the six alprazolam-dependent dogs by administering a liposomal suspension of flumazenil (50 mg/ml) intravenously. The IV infusion of flumazenil did not significantly alter the plasma levels of alprazolam 3 hours after the maintenance dose in dogs stabilized approximately 5–6 weeks in blood collected about 5 minutes after the infusion of flumazenil. In the absence of flumazenil the following levels were estimated: alprazolam, 0.25 ± 0.16 μg/ml; 4-hydroxyalprazolam, 0.47 ± 0.13 μg/ml and alpha-hydroxyalprazolam, 5.79 ± 1.17 μg/ml. In the presence of flumazenil the following levels were obtained: alprazolam, 0.30 ± 0.14 μg/ml and alpha-hydroxyalprazolam, 6.70 ± 1.70 μg/ml, whereas the levels of 4-hydroxyalprazolam were below the level of detection.

Pharmacokinetic measurements show that the half-life of alprazolam (41.4 minutes) in alprazolam-dependent dogs is markedly less than that of its major metabolites 4-hydroxyalprazolam (95.1 minutes) and alpha-hydroxyalprazolam (228.0 minutes). Further, the maximum concentration of alprazolam (C_{max}) in plasma is also markedly less; the time (t_{max}) to reach C_{max} tends to be less and the steady state concentration (C_{ss}) is significantly less than for 4-hydroxyalprazolam and alpha-hydroxyalprazolam (Table 3).

Table 4 compares plasma and brain levels of alprazolam and its metabolites that were obtained in naive and alprazolam-dependent dogs 1 hour after a single dose with levels obtained 1 hour after the morning maintenance dose of 12 mg/kg of alprazolam. As can be seen, there is a statistically significant accumulation of alpha-hydroxyalprazolam but not alprazolam in the plasma and brain after chronic administration. Further, plasma and brain concentrations of both compounds are in equilibrium after either a single dose or chronic treatment with alprazolam whereas 4-hydroxyalprazolam levels were below the level of detection in brain tissue after both a single dose and chronic alprazolam. There may be some accumulation of this compound in plasma but not in the brain after chronic dosing.

DISCUSSION

Chronically administered alprazolam can clearly produce a high level of physical dependence in the dog as evidenced by an abstinence syndrome precipitated with orally administered flumazenil without overt signs of intoxication or of weight loss. The major signs of precipitated abstinence were clonic and tonic-clonic seizures. The incidence of tonic-clonic seizures was dose-related. It has been shown previously that 2 of 4 rhesus monkeys made

dependent on 4 mg/kg of alprazolam administered twice daily for over 4 weeks exhibited convulsions when they were withdrawn (37). One out of four squirrel monkeys who received 2 mg/kg of alprazolam for 18 days had convulsions following the administration of flumazenil intravenously (23).

After the dogs had tonic-clonic seizures they generally proceeded to postictal depression which may have masked some of the signs of precipitated abstinence used in the NPAS scoring system. This could have been a contributing factor to the failure to detect a between-doses effect for the NPAS score since seizure activity increased with the dose of flumazenil. It is highly unlikely that the fact that the lactose placebo was not administered in a Latin square design could have contributed to this lack of between-doses effect. First of all, since it was a constant subtracted from the scores for each dose for each dog, it would not have altered the slope of the dose-response line. Further, Table 5 shows that the NPAS scores for the lactose placebo in the present study where the observers were not blind to the treatment were not statistically significantly different from the values obtained in the flunitrazepam studies where the placebo was administered in a 3×3 Latin square design where the observers were blind to the placebo treatment. The large variance for the flunitrazepam-dependent dogs was due to a high score in one dog. Table 5 also shows that the placebo values in the alprazolam-dependent dogs were higher than the placebo values in the naive dogs where the observers also were not blind to the treatment condition. This is probably due to the effect of alprazolam.

It is known that alprazolam is extensively metabolized in man. However, in the present studies, only alpha-hydroxyalprazolam and 4-hydroxyalprazolam were detectable in dog plasma and of the two metabolites, alpha-hydroxyalprazolam was the major one. This is also the major metabolite in man but it does not accumulate (7, 9, 10). In addition to the alpha-hydroxy- and 4-hydroxy-metabolites, desmethyl-alprazolam (8–10) and alpha-4-dihydroxy-alprazolam (8) are metabolites found in man that were not detected in the dog. Plasma levels of alprazolam and 4-hydroxyalprazolam were low in the dog whereas higher plasma levels of alprazolam but not 4-hydroxyalprazolam have been found in man (8–10, 34, 35). The statistically significantly higher plasma levels of alpha-hydroxyalprazolam found during chronic treatment with alprazolam relative to a single oral dose indicate that this metabolite does accumulate in the dog. On the basis of dosing intervals and half-life in the dependent animal, an accumulation factor of 1.7 was predicted for alprazolam. Our data revealed an accumulation factor of 4.1 for alpha-hydroxyalprazolam whereas neither alprazolam nor 4-hydroxyalprazolam accumulated in the plasma of the dog. Plasma levels of both alpha-hydroxyalprazolam and alprazolam, however, were in equilibrium with brain levels after both single and multiple doses of alprazolam. Most of the precipitated convulsions that occurred both in the crossover study and after intravenously administered flumazenil were manifest between 2 and 3 hours after the last stabilization dose of alprazolam. At this time, plasma levels of alprazolam were quite low whereas plasma levels of alpha-hydroxyalprazolam were 10 to 20 times higher. It has been shown that alprazolam and alpha-hydroxyalprazolam have about equal affinity for binding to crude rat brain membranes and are about equipotent in protecting mice against metrazol- and nicotine-induced seizures and against hypoxic stress (13,29). These data taken together suggest that alpha-hydroxyalprazolam may play a role in the production of alprazolam physical dependence in the dog.

Alprazolam has been estimated to have an affinity for benzodiazepine receptors about two to seven times that of diazepam in the rat brain (2, 15, 21, 30, 31). It has anxiolytic, hypnotic, muscle relaxant and anticonvulsant properties similar to diazepam but is a weaker muscle relaxant and anticonvulsant than diazepam. In animals it appears to be 3 to 5 times more potent and to have a flatter dose-response curve than diazepam (9). In the present study the dose level of alprazolam required to produce physical dependence as measured by flumazenil-precipitated abstinence was 1.3 to 2 times the doses used in diazepam studies for producing physical dependence in the dog. There was no statistically significant difference between alprazolam-dependent dogs in the present studies and a separate group of diazepam-dependent dogs that were studied concurrently [(20), Martin *et al.*, in preparation] in the incidence of convulsions precipitated by flumazenil. It should be pointed out, however, that flumazenil produced a dose-related increase in seizure activity in alprazolam-dependent dogs but not in diazepam-dependent dogs. The NPAS scores for alprazolam-dependent dogs were less ($p<0.1$) for the 6 and 18 mg/kg doses of flumazenil in the alprazolam-dependent dogs than for the diazepam-dependent dogs. Alprazolam-dependent dogs who were precipitated by administering a 5% liposomal suspension of flumazenil intravenously also exhibited a high incidence of clonic and tonic-clonic convulsions along with some of the other signs of abstinence observed in the crossover studies. Some signs of abstinence such as nuchal and limb rigidity were more apparent after IV flumazenil than in the crossover study since the dogs were suspended in a sling in these studies. These results suggest that precipitated abstinence in alprazolam-dependent dogs differs in several respects from that seen in diazepam-dependent dogs and is consistent with the alprazolam withdrawal pattern seen in man which appears to be different from other benzodiazepines (4).

Several investigators have reported that flumazenil itself has intrinsic activity [cf. (12) for review]. In the present study, flumazenil produced some behavioral depression in the naive dog during both the crossover study and during its IV infusion. The only sign of abstinence which was significantly changed, however, was lip licking which was depressed, a sign which is elevated during precipitated abstinence from benzodiazepines (Martin *et al.*, in preparation). Some mild signs of abstinence such as mild rigidity and head and body tremors were observed in some of the dogs after the intravenous infusion of flumazenil. In this regard it has been suggested that the unusual pharmacological profile of flumazenil can be accounted for by assuming that the benzodiazepine function requires the presence of two endogenous ligands with opposing pharmacologic effects, both of which can be antagonized by flumazenil (12). No naive dog treated with a wide range of doses of flumazenil nor any benzodiazepine-dependent dog treated with a lactose placebo had a clonic or tonic-clonic seizure. Thus, the present data do clearly indicate that the signs of flumazenil-precipitated abstinence are not due to either agonistic or inverse agonistic activity of flumazenil itself.

There are several clinical reports of patients who received alprazolam chronically for therapeutic purposes experiencing signs and symptoms of abstinence following withdrawal, including tonic-clonic seizures and myoclonic jerks (3, 16–18, 25), delusions, perceptual distortions and hallucinations (4, 11, 18, 25), agitation, anxiety and tremulousness (11, 16–18, 25), muscle spasms (17) and insomnia (32). Several of these signs or related ones have also been observed in the alprazolam-dependent dogs. This flumazenil-elicited abstinence in the dog is characterized by clonic and tonic-clonic convulsions in the absence of dramatic increases in the NPAS scores. Further, two of the dogs exhibited unique behaviors resembling canine delirium. These two dogs had repeated episodes of wild running and barking, lunging at nonexistent objects, uncontrolled flinging and splaying of the limbs, rigidity and limb jerks. These behaviors were not observed during flumazenil-precipitated abstinence in a large group of dogs dependent on other benzodiazepines (diazepam, nordiazepam, flunitrazepam, oxazepam, halazepam, and lorazepam). Alprazolam does not accumulate in the dog while its major metabolite,

alpha-hydroxyalprazolam, does during chronic administration. Further, the plasma and brain levels of alpha-hydroxyalprazolam are high while alprazolam levels are low during the time-course of peak precipitated abstinence effects, which suggests that alpha-hydroxyalprazolam plays a role in the dependence produced by alprazolam.

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REFERENCES

1. Aden, G. C.; Thein, S. G., Jr. Alprazolam compared to diazepam and placebo in the treatment of anxiety. Clin. Psychiatry 41:245–248; 1980.
2. Braestrup, C.; Squires, R. F. Brain specific benzodiazepine receptors. Br. J. Psychiatry 133:249–260; 1978.
3. Breier, A.; Charney, D. S.; Nelson, C. Seizures induced by abrupt discontinuation of alprazolam. Am. J. Psychiatry 141:1606–1607; 1984.
4. Browne, J. L.; Hauge, K. J. A review of alprazolam withdrawal. Drug Intell. Clin. Pharm. 20:837–841; 1986.
5. Davidson, K.; Farquharson, R. G.; Khan, M. C.; Majid, A. A double blind comparison of alprazolam, diazepam and placebo in the treatment of anxious out-patients. Psychopharmacology (Berlin) 80:308–310; 1983.
6. Dawn Report (Data from the Drug Abuse Warning Network), NIDA Statistical Series annual data, U.S. Department of Health and Human Services, Public Health Service, Alcohol, Drug Abuse and Mental Health Administration, Series 1, #7; 1987.
7. Dawson, G. W.; Jue, S. G.; Brogden, R. N. Alprazolam. A review of its pharmacodynamic properties and efficacy in the treatment of depression. Drugs 27:132–147; 1984.
8. Eberts, F. S.; Philopoulos, Y.; Reineke, L. M.; Vliek, R. W. Disposition of ^{14}C-alprazolam, a new anxiolytic-antidepressant in man. Pharmacologist 22:279; 1980.
9. Evans, R. L. Alprazolam (Xanax, The Upjohn Company). Drug Intell. Clin. Pharm. 15:633–638; 1981.
10. Fawcett, J. A.; Kravitz, H. M. Alprazolam: Pharmacokinetics, clinical efficacy, and mechanism of action. Pharmacotherapy 2: 243–254; 1982.
11. Fernando, L.; Sagi, E. Alprazolam withdrawal syndrome. Can. J. Psychiatry 31:488; 1986.
12. File, S. W.; Pellow, S. Intrinsic actions of the benzodiazepine receptor antagonist Ro 15-1788. Psychopharmacology (Berlin) 88: 1–11; 1986.
13. Gall, M.; Kamdar, B. V.; Collins, R. J. Pharmacology of some metabolites of triazolam, alprazolam, and diazepam prepared by a simple, one-step oxidation of benzodiazepines. J. Med. Chem. 21:1290–1293; 1978.
14. Gibaldi, M.; Perrier, D. Pharmacokinetics. New York: Marcell Dekker, Inc.; 1982.
15. Groh, B.; Muller, W. E. A comparison of the relative in vitro and in vivo binding affinities of various benzodiazepines and related compounds for the benzodiazepine receptor and for the peripheral benzodiazepine binding site. Res. Commun. Chem. Pathol. Pharmacol. 49(3):463–466; 1985.
16. Juergens, S. M.; Morse, R. M. Alprazolam dependence in seven patients. Am. J. Psychiatry 145:625–627; 1988.
17. Kantor, S. J. A difficult alprazolam withdrawal. J. Clin. Psychopharmacol. 6:124–125; 1986.
18. Levy, A. B. Delirium and seizures due to abrupt alprazolam withdrawal: Case report. J. Clin. Psychiatry 45:38–39; 1984.
19. McNicholas, L. F.; Martin, W. R.; Cherian, S. Physical dependence on diazepam and lorazepam in the dog. J. Pharmacol. Exp. Ther. 266(3):783–789; 1983.
20. McNicholas, L. F.; Martin, W. R.; Sloan, J. W.; Wala, E. Precipitation of abstinence in nordiazepam and diazepam dependent dogs. J. Pharmacol. Exp. Ther. 245(1):221–224; 1988.
21. Mackerer, C. R.; Kochman, R. L.; Bierschenk, B. A.; Bremner, S. S. The binding of [^{3}H] diazepam to rat brain homogenates. J. Pharmacol. Exp. Ther. 206:405–413; 1978.
22. Maletzky, B. M. Anxiolytic efficacy of alprazolam compared to diazepam and placebo. J. Int. Med. Res. 8:139–143; 1980.
23. Martin, J. R.; Cumin, R.; Haefely, W. E. Precipitated withdrawal in monkeys after repeated daily administration of different benzodiazepines. Soc. Neurosci. Abstr. 14(2):1109; 1988.
24. Nakajima, R.; Take, Y.; Moriya, R.; Saji, Y.; Yui, T.; Nagawa, Y. Pharmacological studies on new potent central depressants, 8-chloro-6-phenyl-4H-s-triazolo [4-3a] [1,4] benzodiazepine (D-40TA) and its 1-methyl analogue (D-65MT). Jpn. J. Pharmacol. 21:497–519; 1971.
25. Noyes, R., Jr.; Perry, P. J.; Crowe, R. R.; Coryell, W. H.; Clancy, J.; Yamada, T.; Gabel, J. Seizures following the withdrawal of alprazolam. J. Nerv. Ment. Dis. 1:50–52; 1986.
26. Rao, S. N.; Dhar, A. K.; Kutt, H.; Okamoto, M. Determination of diazepam and its pharmacologically active metabolites in blood by Bond Elut column extraction and reversed-phase high performance liquid chormatography. J. Chromatogr. 231:341–348; 1982.
27. Rudzik, A. D.; Hester, J. B.; Friis, W. Pharmacologic activity of a series of 6-phenyl-4H-s-triazolo[4,3-a] [1,4] benzodiazepines in mice. Pharmacologist 13:205; 1971.
28. Rudzik, A. D.; Hester, J. B.; Tang, A. H.; Straw, R. N.; Friis, W. The benzodiazepines. New York: Raven Press; 1973:285–297.
29. Sethy, V. H. Pharmacokinetic studies of triazolobenzodiazepines by drug-receptor binding assays. In: Usdin, E.; Skolnick, P.; Tallman, J. F.; Greenblatt, D.; Paul, S. M., eds. Pharmacology of benzodiazepines. Riverside, NJ: Macmillan Company; 1982:455–462.
30. Sethy, V. H.; Harris, D. W. Benzodiazepine receptor number after acute administration of alprazolam and diazepam. Res. Commun. Chem. Pathol. Pharmacol. 35:229–235; 1982.
31. Sethy, V. H.; Harris, D. W. Determination of biological activity of alprazolam, triazolam and their metabolites. J. Pharm. Pharmacol. 34:115–116; 1982.
32. Slak, S. Alprazolam withdrawal insomnia. Psychol. Rep. 58:343–346; 1986.
33. Sloan, J. W.; Martin, W. R.; Clements, T. H.; Buchwald, W. F.; Bridges, S. R. Factors influencing brain and tissue levels of tryptamine: Species, drugs and lesions. J. Pharmacol. Exp. Ther. 24:523–532; 1975.
34. Smith, R. B.; Gwilt, P. R.; Wright, C. E. Single- and multiple-dose pharmacokinetics of oral alprazolam in healthy smoking and non-smoking men. Clin. Pharm. 2:139–143; 1983.
35. Smith, R. B.; Kroboth, P. D.; Vanderlugt, J. T.; Phillips, J. P.; Juhl, R. P. Pharmacokinetics and pharmacodynamics of alprazolam after oral and IV administration. Psychopharmacology (Berlin) 84:452–456; 1984.
36. Wala, E.; McNicholas, L. F.; Sloan, J. W.; Martin, W. R. Flumazenil oral absorption in dogs. Pharmacol. Biochem. Behav. 30:945–948; 1988.
37. Yanagita, T.; Wakasa, Y.; Kato, S. Dependence potential of alprazolam tested in Rhesus monkeys. Central Institute for Exp. Animals. Preclinical Research Report 7. 2:91–99; 1981.