Pharmacology Biochemistry and Behavior, Vol. 46, pp. 683–688, 1993
Printed in the U.S.A.

0091-3057/93 $6.00 + .00

Precipitated Abstinence in the Diazepam-Dependent Rat

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Received 27 January 1993

MARTIN, W. R., J. W. SLOAN AND E. P. WALA. *Precipitated abstinence in the diazepam-dependent rat.* PHARMACOL BIOCHEM BEHAV 46(3) 683–688, 1993.—Physical dependence was produced in the rat by exposure to continuous release of diazepam from silastic capsule implants (recrystallized diazepam) or by dosing through a gastric fistula. The precipitated abstinence syndrome induced by the IV infusion of flumazenil was characterized by clonic and tonic-clonic seizures, retropulsion, digging, rearing, head, limb and body tremors, twitches and jerks of the body, and ear twitches. This abstinence syndrome differed both qualitatively and quantitatively from the milder syndrome induced in previous experiments by the intragastric administration of flumazenil in the diazepam-dependent gastric fistula rat. Capsule-implanted rats had free plasma and extraneuronal brain levels of diazepam, oxazepam, and nordiazepam in the 10^{-3} to 10^{-4} mg/ml range, and their brain : plasma ratios were not significantly different from 1. The diazepam capsules had a sustained release of over 28 days. These studies show that the capsule implantation technique is an efficacious way of maintaining plasma levels of diazepam and its metabolites, and producing a high level of physical dependence in the rat.

Flumazenil Ro 15-1788 Diazepam dependence in rats Gastric fistula Diazepam capsule implants Precipitated abstinence Flumazenil plasma levels, rat Diazepam plasma levels, rat

SEVERAL investigators have shown that rats can be made physically dependent on benzodiazepines; however, signs of withdrawal abstinence were mild and did not include tonic-clonic seizures (3,4,10,14). The benzodiazepine antagonist, flumazenil (Ro 15-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazol[1,5*a*][1,4]benzodiazepine-3-carboxylate), has been shown to precipitate abstinence in the diazepam-dependent rat (133 mg/kg/day administered in four equally divided doses intragastrically). However, even when very large doses of flumazenil (up to 120 mg/kg) were administered intragastrically (IG), only a mild abstinence syndrome was precipitated (6). The maximum intensity of precipitated abstinence was less than that observed following withdrawal abstinence (3). A procedure for administering diazepam in silastic capsules implanted subcutaneously (SC) in rats has been developed (2). Further, it has been shown that tonic-clonic seizures can be precipitated by intravenously (IV) infusing flumazenil, suspended in carboxymethylcellulose, in rats made dependent on implanted diazepam-containing silastic capsules (13). The studies reported here present additional results on precipitated abstinence in rats made dependent on diazepam using this technique (2,13). It further compares plasma and brain levels of diazepam and its metabolites, and the ability of IV administered flumazenil to precipitate abstinence signs in rats made dependent on diazepam administered by capsule implantation and by IG administration.

METHOD

The methods employed in this study, except for minor modifications, have been previously described (2–4,8). Female Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing between 206 and 290 g were used. Capsules prepared from silastic tubing sealed at both ends (Silastic Medical Adhesive, Silicone Type A, Dow-Corning: Dow-Corning Medical Grade Silastic Tubing, i.d. 0.058 × o.d. 0.077 in.) containing 90 mg of diazepam were implanted in the backs of donor rats. After 1 day, two capsules were transferred to the backs of experimental rats from donor rats. Additional conditioned capsules were implanted at 10-day intervals after the initial implantation. Precipitation studies were initiated 16 to 22 days after the first implantation of diazepam capsules or 8 to 15 days after the implantation of lactose placebo capsules in one series of experiments (series A) and 31 to 36 days after implantation of diazepam capsules in another series of experiments (series B). Abstinence was precipitated by infusing flumazenil (0.5 mg/ml) suspended in a 0.2% solution of carboxymethylcellulose containing 0.1% Tween 80 IV at a rate of 1 ml/min for

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20 min in seven rats using an indwelling 24-ga Quik-Cath cannula (Travenol) placed in a tail vein (series A). Following the implantation of the tail vein cannula, the rats were placed in a circular observation chamber (20 in. in diameter and 8.5 in. high) with a sawdust-covered floor. Immediately after the rat was placed in the chamber, the infusion of flumazenil was begun.

In series B, seven rats anesthetized with ketamine and acepromazine were implanted with a jugular polyethylene (PE 10) cannula that was exteriorized in the dorsal nuchal midline. The following morning, the rats were connected to an infusion pump and were observed during two 5-min intervals. Following the predrug observation periods, an infusion of flumazenil (25 mg/ml) dissolved in dimethyl sulfoxide (DMSO) at a rate of 1 ml/min was begun and continued for 20 min.

In a third series of experiments (series C), gastric fistula rats were prepared and made dependent on diazepam using previously described procedures (4,6,8). Diazepam was administered to these rats through the gastric fistula in #5 gelatin capsules. The dose was escalated to a stabilization dose of approximately 160 mg/kg/day administered in four equally divided doses at approximately 6-h intervals. These rats were treated chronically with diazepam for 8 to 24 days and then IV infused with the suspension of flumazenil in carboxymethylcellulose as described in Experiment A when they had reached the stabilization dose of diazepam. The animals were housed separately with free access to food and water.

In a fourth series of precipitation studies (series D), three rats were made dependent using diazepam capsules as described above but were precipitated with flumazenil dissolved in DMSO administered in an IV (tail vein) bolus starting 5 weeks after the implantation of the first capsules. Three doses of flumazenil were employed (10, 20, and 40 mg/kg using a DMSO solution of 25 mg/ml), administered at weekly intervals using a Latin square design.

Rats in study A and B were observed for 10 min prior to the infusion, for 20 min during the infusion, and for the 10- or 15-min postinfusion period. In study A, five rats that had been implanted with lactose-containing capsules were infused in a similar manner with the carboxymethylcellulose suspension of flumazenil. In study B, four naive rats that had a jugular cannula were infused with a DMSO solution of flumazenil. Rats in study C were observed during the 20-min infusion and a 30-min postinfusion period. Rats in study D were observed for 10 min before the injection of the DMSO flumazenil bolus and for 40 min after injection.

The rats were free to move around the chamber. One observer (JWS or WRM) identified the signs of abstinence while another observer recorded the observations on a checklist whose items are presented in Table 1. This checklist contains three groups of items: a) items that are related to activity. These items are weighted such that all contribute approximately equally to a score that is related in a dose-related way to the depressant effects of sedative-hypnotics (8). A positive score indicates the degree of behavioral activation while a negative score measures depression. b) Weighted items that make an equal contribution to the Precipitated Abstinence Score (PAS) and are related in frequency of occurrence to the dose of flumazenil that was administered orally. c) The third group of items consists of other signs of withdrawal and precipitated abstinence. This form and its use have been previously described (6,8). In the present study, the signs that occurred were tabulated at 5-min intervals for the 20-min period during the infusion of flumazenil and for 10 to 30 min thereafter.

TABLE 1

SIGNS OF PRECIPITATED ABSTINENCE SEEN IN RATS MADE DEPENDENT BY IMPLANTING DIAZEPAM CAPSULES AND THE WEIGHT GIVEN TO SOME OF THESE SIGNS FOR CALCULATING THE PRECIPITATED ABSTINENCE SCALE (PAS)

Sign	Weight
Convulsive Phenomena	
Twitches and jerks	0.8
Clonic convulsions	0.6
Tonic-clonic convulsions	13.3
Other Motor Signs	
Head and body tremors	1.0
Jumping	2.0
Turning	2.2
Backing	0.6
Dyskinesias	
Writhing	0.7
Affective	
Arched back	1.4
Vocalization	1.3
Autonomic	
Respiratory rate	0.2
Other Precipitated Abstinence Signs	
Chewing	
Digging	
Ear twitches	
Explosive awakening or jumping	
Head bobbing	
Hotfoot behavior	
Poker tail	
Rigid walking	
Scratching	

The in vitro release rate of diazepam from capsules formulated from different size particles of diazepam was determined in duplicate or triplicate. Capsules were prepared according to previously described procedures (2) from either untreated, ground and sieved, or recrystallized diazepam that had been ground in a ball mill for 20 min. The sieving was done using stacked screens (0.0059-, 0.0029-, and 0.0017-in. openings) and a receptacle. The screen stack was loaded with the milled diazepam and shaken for 20 min. The drug on each screen and in the receptacle was retrieved and used to prepare capsules that were appropriately designed. The in vitro release rate from duplicate or triplicate capsules containing the various-sized particles was determined by placing them in separate scintillation vials and completely covering them with 20 ml of normal saline. The scintillation vials were placed in a temperature-controlled (37°C) incubator and shaken constantly for 24 h. Capsules were then transferred to a vial containing 20 ml of fresh normal saline. This procedure was repeated for 28 days. Aliquots (20 μl) of the bathing solution collected daily were diluted to 40 μl with an internal standard (flunitrazepam, 20 μg/ml in methanol). The amount of diazepam released daily was determined in 5 μl of this solution by HPLC (9). In vivo plasma levels of diazepam and its metabolites were also determined using the same HPLC method (9) in blood obtained from the rat tail vein.

TABLE 2

SUMMARY OF THE FLUMAZENIL CARBOXYMETHYLCELLULOSE AND DMSO INFUSION SEIZURE DATA IN RATS MADE DEPENDENT ON DIAZEPAM USING THE CAPSULE IMPLANT AND GASTRIC FISTULA TECHNIQUES

Time Dosed or Implanted (days)	Flumazelil Dose (mg/kg)	Number of Clonic Seizures	Number of Tonic-Clonic Seizures
	Series A		
Diazepam Implant – Flumazenil Carboxymethylcellulose Infusion			
18	51.6	1	1
19	42.7	0	0
22	46.1	1	0
21	48.1	1	0
22	48.5	2	1
16	39.7	1	0
19	44.4	3	0
	45.9 ± 1.5	6/7	2/7
Diazepam Implant – Carboxymethylcellulose – Vehicle Infusion			
16	0	0	0
17	0	0	0
16	0	0	0
19	0	0	0
18	0	0	0
17	0	0	0
	0	$0/6^{0.005}$	0/6
Lactose Implants – Flumazenil Infusion			
8	36.8	0	0
8	37.0	0	0
7	37.4	0	0
14	34.5	0	0
14	37.3	0	0
15	37.9	0	0
	36.8 ± 0.8	$0/6^{0.005}$	0/6
	Series B		
Diazem Implant – Flumazenil DMSO Infusion			
35	144.8	4	0
33	127.4	28	0
36	179.8	12	0
31	228.7	0	0
31	191.6	91	7
34	176.7	53	0
35	184.8	27	3
	176.3 ± 12.4	6/7	2/7
Naive Rats – Flumazenil DMSO Infusion			
No implant	160.5	0	1
	174.2	12	0
	172.4	0	0
	179.9	0	0
	172.2 ± 7.2	1/4	1/4
	Series C		
Gastric Fistula Diazepam Dependent – Flumazenil CMC Infusion			
19	29.1	0	1
19	44.8	0	0
21	40.2	0	0
33	50.0	0	0
33	39.4	1	1
35	40.5	0	0
12	79.4	1	1
	46.2 ± 6.0	$2/7^{0.05}$	3/7

Each parameter and observation set is for independent groups. Superscripts indicate p values of differences between the appropriate control data and the diazepam-dependent implant or gastric fistula flumazenil experiment. These observations were made in 26 rats. The mean total dose of flumazenil infused ± SE is also presented.

Microdialysis of Brain Tissue and Plasma

CMA/10 microdialysis probes (3 mm tip, 20 mm cannula, 0.65 o.d., molecular cut off below 20000 Da), CMA/100 microinjection pump, and CMA/140 microfraction collector were obtained from Carnegie Medicin BAS (West Lafayette, IN). Rats used in these experiments were from study B.

The rats were anesthetized with sodium pentobarbital (45 mg/kg, IP) and placed in a stereotaxic instrument. A part of the calvarium was removed and a flap of dura over the parietal cortex was turned back. The microdialysis probe was implanted stereotaxically 5.8 mm deep into the brain cortex, 3.8 mm anterior and 2 mm lateral to the bregma. Before and during the implantation procedure, the probe was continuously perfused with artificial cerebrospinal fluid (126.5 mM NaCl, 27.5 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO, 1.1 mM $CaCl_2$, 0.85 mM $MgCl_2$, 0.5 mM Na_2SO_4, and 5.9 mM glucose, pH 7.5) at a rate of 10 μl/min using the microinjection pump. Following implantation of the probe, the flow rate was reduced to 2 μl/min and was maintained at this rate throughout the microdialysis experiment. After a 30-min equilibration period, the perfusates from the probe were

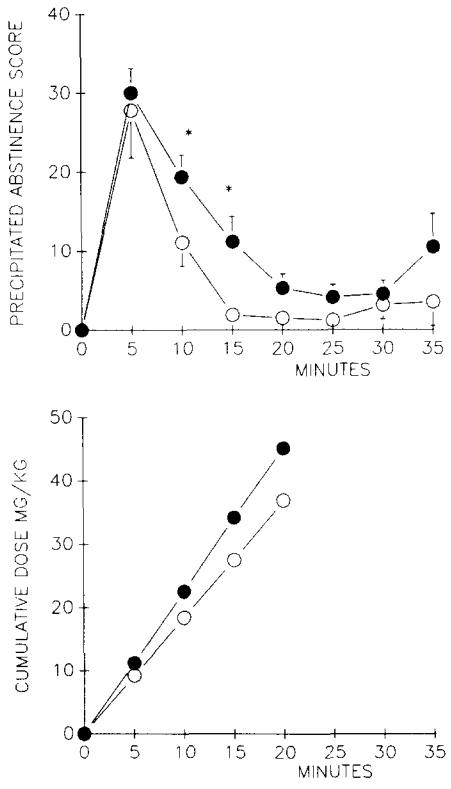

FIG. 1. Precipitated abstinence scores evoked by a 20-min infusion of a carboxymethylcellulose suspension of flumazenil (0.2%) in diazepam (●) and lactose (○) capsule-implanted rats are presented in the upper panel. Seven rats were implanted with diazepam capsules and with lactose capsules. Each point with associated bar is a mean and SE. Up bars are for diazepam-dependent rats, down bars are for lactose capsule-implanted rats. Significant differences ($p < 0.05$) between the diazepam- and lactose-treated rats are indicated by an asterisk. The cumulative amount of flumazenil administered is presented in the lower panel.

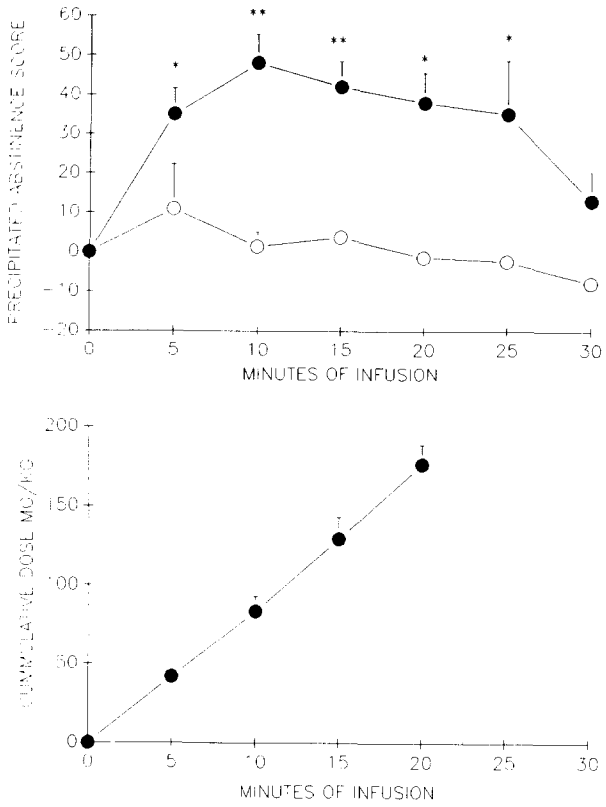

FIG. 2. Precipitated abstinence scores evoked by a 20-min infusion of a DMSO flumazenil solution (2.5%) in diazepam capsule-implanted (●) and naive (○) rats are presented in the upper panel. Each point and associated bar is the mean and SE of determination made in seven diazepam-dependent rats and four naive rats. The cumulative amounts of flumazenil administered are presented in the lower panel. $*p < 0.05$; $**p < 0.01$.

collected for 2 h at 25-min time intervals and analyzed immediately for the levels of oxazepam, nordiazepam, and diazepam.

Following removal from the brain, the microdialysis probe was washed by perfusing with distilled water for 30 min at a rate of 10 μl/min. Afterward, the probe was immersed into conical vials filled with plasma and perfused at room temperature with isotonic solution of phosphate buffer (80.9 mM K_2HPO_4, 18.4 mM KH_2PO_4, 34.1 mM NaCl). After a 30-min stabilization period, three samples of the plasma perfusates were collected at 25-min time intervals and analyzed for the drug levels. The concentrations of oxazepam, nordiazepam, and diazepam in the collected perfusates were related to that in the fluids surrounding the microdialysis probes by the efficiency of recovery of each probe.

The recovery of diazepam, nordiazepam, and oxazepam across the microdialysis membrane was determined for each probe after use. This was accomplished by placing the tip of the microdialysis probe into 1 μg/ml solution of oxazepam, nordiazepam, and diazepam mixture, perfusing at 2 μl/ml, collecting samples of the perfusates, and determining the drug concentrations in the perfusates and the outer media. Standard curves for the drugs were prepared for the concentrations ranging from 5 ng/ml to 10 μg/ml. The mean relative recoveries of oxazepam, nordiazepam, and diazepam across microdialysis probes were equal to 22.6 + 3.9%, 16.0 + 1.5%, and 13.7 + 1.4%, respectively.

At the end of the experiment, the animals were decapitated, trunk blood was collected into EDTA tubes, and the brain was removed rapidly. Plasma and brain tissue were stored at −70°C before analysis for the total levels of diazepam and its metabolites.

The concentrations of diazepam, nordiazepam, and oxazepam in plasma, brain tissue, and plasma and brain perfusates were determined by HPLC as described previously (11,12). Briefly, the brain tissue was homogenized with ice-cold 0.9% NaCl (1 : 4 w/v). Buffered (pH 9.4) Bond Elut C-18, 1 cc and 3 cc columns, were used for a solid phase extraction of plasma and brain homogenates, respectively. Flunitrazepam (50 ng injected) was used as an internal standard. Standard curves for oxazepam, nordiazepam, and diazepam extracted from plasma and brain homogenates were linear over the range 2.5 to 250 ng, with a limit of sensitivity of 1.25 ng. The recovery ranged from 69% to 83% with day-to-day coefficient of variation lower than 3% and 13% for plasma and brain, respectively. The levels of diazepam, nordiazepam, and oxazepam in microdialysis perfusates were determined by direct injection of 50-μl samples (mixture of 50 μl perfusate and 10 μl of 25 μg/ml methanol solution of flunitrazepam) on HPLC column using 50-μl pressure-lok liquid syringe (Dynatech Precision Sampling Co.). The external standards were analyzed along with each set of the unknown samples.

RESULT

The release rates of several capsule preparations were evaluated employing different diazepam particle sizes. There was a trend for capsules made of smaller particles (≤0.0017 in.) to release diazepam in vitro more rapidly than unscreened diazepam and capsules made with larger particle sizes. When diazepam was mixed with absolute alcohol, dried and ground in a ball mill prior to formulating (recrystallized) (2), the silastic capsule so formulated had the highest release rate. This formulation was used in all diazepam capsule dependence studies. Although the release rate varied somewhat from day to day, it did not show a decrement over a 28-day period. The mean rate constant was 0.84 mg/day. These studies of release rate employed procedures previously described (2), except that the release was determined by measuring the concentration of the diazepam in the bathing media using HPLC rather than determining the release of isotopically labelled diazepam. Although these release rates are somewhat greater than those reported previously (2), they confirm the observation that the release rate is relatively constant over a 28- to 30-day period.

In study A, a carboxymethylcellulose suspension of flumazenil (0.5 mg/ml) was infused for 20 min at a rate of 1.0 ml/min. This experiment was controlled by infusing the carboxymethylcellulose vehicle in diazepam-dependent rats and by infusing the carboxymethylcellulose flumazenil suspension in seven rats implanted with lactose capsules. These results are summarized in Table 2 and in Fig. 1. The diazepam capsule-dependent rats receiving a carboxymethylcellulose flumazenil infusion had significantly more, and a higher incidence of, clonic seizures when compared to the lactose capsule-implanted control group and the carboxymethylcellulose flumazenil-infused gastric fistula rats. The combined diazepam capsule- and gastric fistula-dependent rats in the series A and C infu-

TABLE 3

CLONIC (C) OR TONIC-CLONIC (T-C) SEIZURES INDUCED BY GRADED DOSES OF FLUMAZENIL ADMINISTERED BY A BOLUS IV TAIL VEIN INJECTION IN THREE DIAZEPAM-DEPENDENT RATS (90 MG/CAP/WEEK)

	Series D Dose of Flumazenil					
	10 mg/kg		20 mg/kg		40 mg/kg	
Rat #	C	T-C	C	T-C	C	T-C
1	0	0	0	0	0	0
2	0	0	0	0	0	6
3	0	0	1	2	0	3
Total seizures	0 (0/3)*	0 (0/3)	1 (1/3)	2 (1/3)	0 (0/3)	9 (2/3)

*Values in parentheses = total number of rats that had seizures/total number of rats in group.

sion experiments had significantly more tonic-clonic seizures than the combined control carboxymethylcellulose-infused diazepam-dependent groups (5/14 vs. 0/12). The seizures had a latency of 0.86 to 52 min following the onset of the carboxymethylcellulose infusion. Figure 1 compares the Precipitated Abstinence Scores (PAS) in the diazepam-dependent and the lactose capsule-implanted rats during flumazenil infusions. As can be seen, the PASs for both the diazepam and lactose capsule-implanted rats were both elevated 5min after the onset of infusion and the means were not significantly different. Differences between the diazepam and the lactose capsule-implanted rats were significant only at the 10- and 15-min observations, at which times the diazepam-treated rats had the higher scores.

Although the number and proportion of dependent rats having convulsions in study B were the same as in study A, the rats in study B had more convulsions (Table 2 and Fig. 2). Further, the flumazenil-DMSO solution produced no significant changes in PAS in naive rats, and all PASs of the diazepam-dependent rats were significantly higher than those of the naive rats during the infusion period and immediately thereafter (Fig. 2). As can be seen from Table 2, one naive rat exhibited clonus at the end of the flumazenil infusion and another a tonic-clonic convulsion during 5 to 10 min after the beginning of the flumazenil infusion.

Table 3 presents the results of dose ranging (study D) in which the flumazenil was administered as an IV bolus. Higher doses of flumazenil could produce tonic-clonic seizures followed by death. As can be seen, the incidence of tonic-clonic seizures was dose-related. These seizures came on rapidly and were followed by postictal depression that precluded the emergence of other signs of abstinence.

The most common signs of precipitated abstinence observed following CMC and DMSO infusion are presented as weighted signs. These signs were weighted such that they made approximately equal contributions to the Precipitated Abstinence Score. Thus, the smaller the weighting factor, the more common the sign.

Table 4 presents the free (B_f and P_f) and total (B_t and P_t) concentrations of diazepam and its metabolites in brain (B) and plasma (P). The free plasma concentrations of DZ, ND, and OX are in equilibrium, as indicated by the fact that the B_f/P_f ratios are not significantly different from 1. Although total brain and plasma levels of DZ, ND, and OX are not particularly high, free levels are relatively high, suggesting low protein binding in the rat.

TABLE 4

LEVELS (μg/ml) AND DISTRIBUTION OF OXAZEPAM, NORDIAZEPAM, AND DIAZEPAM BETWEEN PLASMA AND BRAIN IN DIAZEPAM-DEPENDENT RATS

	DZ	ND	OX
Extraneuronal brain space (B_f)	0.543 ± 0.09	0.421 ± 0.081	0.321 ± 0.055
Plasma (P_f)	.545 ± .068	.334 ± .051	.262 ± .027
Brain tissue (B_t)	2.305 ± .579	.921 ± .221	.440 ± .087
Plasma (P_t)	$1.409^{0.001}$ ± .137	$.466^{0.001}$ ± .08	$.653^{0.001}$ ± .071
B_f/P_f	1.013 ± .158	1.119 ± .159	1.291 ± .192
B_t/P_t	1.604 ± .304	$2.045 \pm .315_{0.01}$	$.715 \pm .120_{0.05}$

Data are means ± SE of determinations made in 15 rats. Superscripts indicate the significant p values for the paired t comparison between plasma and brain levels. Subscript indicates the p value of difference from 1.

DISCUSSION

These results confirm and extend previous observations (5,12) showing that rats can be made dependent by administering diazepam-containing capsules IG and by implanting silastic capsules containing diazepam SC, as revealed by a flumazenil-evoked precipitated abstinence syndrome. DMSO solutions of flumazenil rarely produced signs of precipitated abstinence in naive rats. Several factors probably influence the intensity of the precipitated abstinence syndrome. The intensity of the precipitated abstinence syndrome was much greater in the DMSO (series B) than in the carboxymethylcellulose-infused rats (series A and C) and had a more rapid onset. The abstinence syndrome was clearly manifest within 5 min of infusion and was maximal after 10 min. These experiments differed in several respects. The period of dependency of the DMSO-infused rats (series B) was approximately 2 weeks longer than the carboxymethylcellulose-infused rats (series A). It has been previously shown (13) that the intensity of precipitated abstinence increases with an increase in the duration of dependency. The intensity of the flumazenil precipitated abstinence observed in study B was more severe than was observed in study A and in the rats dosed IG with diazepam. The intensity of the precipitated abstinence is also dependent on the dose of flumazenil (7,5). As can be seen by comparing the bottom panel of Figs. 1 and 2, the DMSO-infused rats received a dose of flumazenil nearly four times that of CMC-infused rats. Hence, the more intense precipitated abstinence syndrome observed in study B is undoubtedly due in part to the longer period of dependence, to the larger amount of flumazenil infused, and its more rapid infusion. The low water solubility of flumazenil limits the amount that can be rapidly administered in either aqueous solution or aqueous suspension; however, this limitation seems to be circumvented with the use of DMSO solutions where effective tissue concentrations can be rapidly achieved. Further, DMSO may have enhanced the permeability of flumazenil through the blood-brain barrier (1). Whereas the tail vein infusion of carboxymethylcellulose is accompanied by abstinence signs other than convulsions, these signs are seen less frequently with DMSO alone. The sites of the cannulae (tail vein vs. jugular vein) and the volume of carboxymethylcellulose infused may have played a role in some of these signs being evoked.

The capsule implantation technique yielded surprisingly high free concentrations of diazepam and its metabolites in both plasma and the brain extraneuronal compartments. The concentrations of free oxazepam and nordiazepam were as high or higher than those observed in orally dosed dogs receiving 9 mg/kg/day administered in three equally divided doses (Wala et al., in preparation). Free diazepam plasma and brain levels were about five times higher in these rats than in dogs receiving 9 mg/kg/day of diazepam. Total plasma and brain levels were several times higher in the dog than in the rat, however. These data suggest that protein binding is less in the rat than in the dog, while the dog accumulates bound nordiazepam more than the rat.

Benzodiazepine withdrawal and precipitated abstinence have been characterized in the rat (6,8) and dog (5) using different procedures for producing dependence and precipitating abstinence. Different syndromes have emerged as the level of dependence has varied; as the dose and vehicle by which flumazenil has been administered has differed; as procedures for making observations have differed and according to whether precipitated or withdrawal abstinence has been studied. The most definitive and severe abstinence syndromes have been observed using capsule implantation for making rats dependent by administering a DMSO solution of flumazenil intravenously.

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