Pergamon

Pharmacology Biochemistry and Behavior, Vol. 48, No. 2, pp. 397–401, 1994

0091-3057/94 $6.00 + .00

0091-3057(93)E0050-E

Chlordiazepoxide, but Not Bretazenil, Produces Acute Dependence, as Evidenced by Disruptions in Schedule-Controlled Behavior

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Received 12 July 1993

BRONSON, M. E. *Chlordiazepoxide, but not bretazenil, produces acute dependence, as evidenced by disruptions in schedule-controlled behavior.* PHARMACOL BIOCHEM BEHAV **48**(2) 397–401, 1994.—The purpose of the present study was to determine whether the full benzodiazepine (BDZ) agonist chlordiazepoxide (CDAP) and the partial BDZ agonist bretazenil would produce acute dependence in rats, as evidenced by disruptions in fixed-interval responding during precipitated abstinence withdrawal. Doses of CDAP and bretazenil administered acutely were 10, 75, and 100 mg/kg; flumazenil (1–56 mg/kg) was administered 1, 2, 4, or 18 h later. Withdrawal, defined as a significant decrease in fixed-interval responding, was only seen when a high dose of flumazenil was administered 18 h after 100 mg/kg of CDAP. These results support those of others (5) who found that high (75–450 mg/kg) doses of CDAP were required to produce acute physical dependence. That bretazenil did not produce acute physical dependence supports the findings of others (20,23) who report that chronic administration of bretazenil does not result in physical dependence.

Chlordiazepoxide Bretazenil Acute dependence Schedule-controlled behavior Rats

BENZODIAZEPINES (BDZs) are widely prescribed psychotropic drugs that have a variety of effects, including sedative, anticonvulsant, and anxiolytic activity. In addition to their therapeutic use, BDZs have also been associated with polydrug abuse among addicts (30), and it is well established that physical dependence develops upon chronic exposure to BDZs. Physical dependence is usually defined as the occurrence of observable signs of withdrawal following termination of long-term drug administration. Although the time course and severity of withdrawal depend upon the pharmacokinetics of the particular BDZ [see (18) for a review], the observable manifestations of BDZ withdrawal are relatively mild. However, more severe symptoms can occur when withdrawal is precipitated with a BDZ antagonist, such as Ro15-1788 (19). Although the observation of physiological signs such as piloerection, arched back, tail erection, etc. does provide a way to measure withdrawal in the rodent (5), there are other behavioral measures that might provide a more sensitive indicator of dependence (26). For example, several studies have shown that schedule-controlled behavior is disrupted in animals following termination of chronic treatment with a variety of drugs (3,6,11,14,27,28,32). Moreover, disruption of schedule-controlled responding is thought to be a particularly sensitive indicator of dependence, since this phenomenon occurs during the early stages of both opioid (2,14,22) and BDZ withdrawal (28,31). In addition, disruption of operant behavior has been observed under conditions that were insufficient to induce observable signs of withdrawal. One way to quantify disruption due to withdrawal is to examine the effects of an antagonist before and after chronic treatment with the agonist. Previous work within the opioid system indicates that animals become more sensitive to the effects of an antagonist as the result of chronic opioid administration. This increased sensitivity, as evidenced by a leftward shift in the antagonist dose-effect curve, has been used as a measure of dependence and an indicator of withdrawal (10,16,34).

Although physical dependence is generally associated with chronic exposure to BDZs, some recent studies show that physical dependence can occur after a single dose of a BDZ (5,25,33). Although overt physical signs of withdrawal were used as a measure of acute BDZ dependence in these studies, at least one investigator (28) has used disruptions in operant behavior to measure acute BDZ dependence. In the latter study in squirrel monkeys, while overt signs of withdrawal

were absent, fixed-interval responding was disrupted when flumazenil was given 1 day after a single, 10-mg/kg dose of chlordiazepoxide.

Acute dependence studies have been used increasingly for a number of reasons: 1) They provide a rapid means of determining the role of various neurotransmitters in the development of tolerance and/or dependence (21,24), 2) they can be used to compare antagonist potencies in precipitating withdrawal (15), 3) they provide a model for testing drugs that might be useful in the treatment of withdrawal (9), and 4) they may provide a means of assessing the ability of novel drugs to produce dependence (4). The current study was conducted to examine the behavioral effects of flumazenil following single doses of CDAP and bretazenil.

Bretazenil, previously referred to as Ro16-6028, is a potent anxiolytic and anticonvulsant, but unlike CDAP and other full BDZ agonists, it has virtually no sedative activity, except at very high doses (12). Chronic treatment with bretazenil has not been shown to produce physical dependence in mice (23) or in squirrel monkeys (20). It should be noted, however, that schedule-controlled behavior has not been used to examine withdrawal from bretazenil. Furthermore, withdrawal due to acute and chronic dependence may be expressed differently (5). It was of interest, therefore, to see if withdrawal, as evidenced by disruptions in schedule-controlled behavior, would occur after a single dose of bretazenil and to compare its effects to those of CDAP.

In precipitated withdrawal studies, the dose of the agonist, the dose of the antagonist, and the time interval between administration of the agonist and antagonist have been shown to be critical (4,8,13,17). The present study examined the effects of various doses of flumazenil given at several different times after a wide range of doses of either bretazenil or CDAP.

METHODS

Subjects

Thirty experimentally naive male Long-Evans hooded rats were used. The rats were approximately three months of age at purchase and were allowed to free-feed for two weeks. They were then weighed and slowly reduced to 80% of their free-feeding weights for a final weight range of 300–320 g. All rats were housed individually with unlimited access to water in a colony maintained on a 12-h light–dark cycle.

Apparatus

Eight standard operant conditioning rat chambers were used. Each chamber was equipped with two centrally mounted 5-cm-long response levers located on the front wall 9 cm from the chamber floor and 3 cm from the side walls. When operated, a pellet dispenser delivered a 45-mg Noyes food pellet (P.J. Noyes Co., Lancaster, NH) into a pellet trough that was centrally mounted between the two levers and approximately 1 cm above the chamber floor. Located above each lever were three stimulus lights. Houselights were centrally mounted 1 cm below the ceiling on the front wall. All chambers were equipped with an exhaust fan that supplied ventilation and white noise that served to mask extraneous sounds. Scheduling of experimental events and data collection were accomplished through the use of a mini computer with Med-State interfacing and software.

Behavioral Procedure

All of the rats were trained to respond on one lever under a fixed-interval 1-min (FI 1′) schedule of food presentation with a limited hold of 10 s (LH 10″). In the FI component, the first response within 10 s after the 60-s interval produced a food pellet and started a 2-s timeout in which the chamber was dark and responses had no scheduled consequences. The timeout period started automatically without food presentation if a response was not made within 10 s after the 60-s interval had elapsed. Training sessions lasted until 30 reinforcers had been earned. Testing was initiated when responding had stabilized, such that there was not more than a 5% variation in day-to-day response rates.

Precipitated Withdrawal Test Procedure

During testing for precipitated withdrawal the session was divided into five components. In each component the FI was in effect for 10 reinforcers and/or limited holds. In the first component, labeled *Baseline* in Table 1, animals received no injection prior to being placed in the operant chambers. In the cases of the 1-, 2-, and 4-h tests, animals were injected immediately after the baseline component and returned to their home cages. In the case of rats to be tested at 18 h, food was given immediately after the baseline component, and injections of bretazenil, CDAP, or water were administered 6 h later. This was done so that the rats would eat all of their food at the normal feeding time and therefore be in the same state of food deprivation when tested at their usual run time the following day. The next four components occurred 1–24 h after the first component. Injections in these components (i.e., components 2–5) were as follows: Rats were injected with water 15 min before component 2. The water injection served as a posttreatment measure of behavior. Immediately after components 2, 3, and 4, rats were injected with successive doses of flumazenil or an equivalent volume of flumazenil vehicle before being returned to the home cages for a 15-min timeout. Rats were fed after the last component. Sessions were conducted seven days a week and tests were separated by at least three days. The doses of CDAP and bretazenil administered acutely were 10, 75, and 100 mg/kg. A dose of 150 mg/kg CDAP was given to 15 animals, but 6 of them died within 48 h, so this dose of both CDAP and bretazenil was dropped from further study. Because of significantly decreased responding in the operant chamber, the only dose of CDAP that could be tested for precipitated withdrawal at 1, 2, and 4 h was 10 mg/kg. CDAP, at 75 and 100 mg/kg, was therefore tested at 18 h, after the animals had fully recovered from the sedative effects of the drug. Bretazenil, on the other hand, did not decrease responding; therefore, all doses of bretazenil were tested with cumulative doses of flumazenil at 1, 2, 4, and 18 h. In addition, because rates of responding were similar to baseline 18 h after CDAP 100 mg/kg, but subsequent administration of flumazenil decreased responding below baseline, CDAP 100 mg/kg was administered a second time and 18 h later all rats received four injections of flumazenil vehicle to determine whether the decrease in responding was due to a vehicle effect after this high dose of CDAP.

Flumazenil vehicle and the four different dose ranges of flumazenil were also tested in water-pretreated animals. To control for a possible effect of time of day (i.e., increased food deprivation), these tests were performed at different times, similar to the precipitated withdrawal test times. Testing with cumulative doses of flumazenil was as follows: Flumazenil, 1, 10, and 32 mg/kg, was administered to animals

TABLE 1

EFFECTS OF FLUMAZENIL OR FLUMAZENIL VEHICLE ON RATES OF RESPONDING IN RATS TREATED WITH EITHER CHLORDIAZEPOXIDE (C), BRETAZENIL (B), OR WATER

Drug Dose-mg/kg (posttreatment test time)	*n*	Component 1—Baseline	Component 2—Posttreatment	Component 3—Flumazenil
				(1 mg/kg)
Water (2)	24	43.3 (4.4)	44.5 (4.0)	44.1 (3.8)
Water (4)	30	47.6 (3.8)	48.6 (3.4)	45.3 (2.9)
C-10 (1)	12	45.3 (4.4)	52.3 (6.3)	47.6 (5.9)
C-10 (2)	6	43.0 (9.6)	53.8 (9.4)*	50.7 (8.6)
C-10 (4)	7	49.6 (7.0)	58.1 (8.7)	47.3 (8.7)
B-10 (1)	12	41.1 (4.7)	54.4 (7.3)*	51.4 (7.4)
				(10 mg/kg)
Water (1)	30	42.2 (3.9)	45.6 (3.6)	39.3 (4.0)
C-75 (18)	12	43.0 (5.3)	51.8 (7.5)	32.8 (6.1)
B-10 (1)	8	40.4 (6.6)	57.0 (7.8)*	47.6 (6.4)
B-10 (2)	8	41.3 (7.6)	41.1 (6.2)	43.3 (6.7)
B-10 (4)	8	43.1 (8.8)	49.0 (10.0)	49.4 (9.9)
B-75 (1)	6	38.7 (3.9)	60.5 (10.3)*	41.3 (6.8)
B-75 (2)	8	51.4 (9.6)	57.9 (8.2)	55.0 (8.3)
B-75 (4)	8	44.3 (7.9)	50.9 (6.7)	50.0 (8.6)
B-100 (1)	8	39.9 (5.6)	42.5 (7.9)	42.0 (5.2)
B-100 (2)	8	47.0 (7.6)	53.0 (13.2)	47.4 (10.9)
B-100 (4)	8	40.8 (6.3)	49.8 (6.2)*	47.1 (7.0)
				(32 mg/kg)
Water (18)	14	40.9 (4.5)	38.3 (5.2)	38.8 (5.8)
C-100 (18)	14	44.4 (4.5)	44.7 (6.5)	28.4 (6.0)*
B-100 (18)	6	43.2 (6.1)	45.3 (4.9)	43.3 (6.2)
				(56 mg/kg)
Water (18)	6	54.3 (13.1)	50.3 (11.6)	55.0 (14.3)
B-75 (18)	6	58.2 (14)	46.0 (11.6)	52.8 (11.7)
				(Flumazenil vehicle)
Water (4)	29	43.5 (3.7)	46.7 (3.7)	44.6 (3.4)
C-100 (18)	14	44.3 (4.6)	41.1 (3.9)	40.6 (4.3)

The first column represents the drug and dose followed by the number of hours that elapsed before testing with flumazenil—that is, C-100 (18) refers to treatment with 100 mg/kg chlordiazepoxide, tested 18 h later with flumazenil. The second column gives the number of animals per group. "Component 1—Baseline" refers to mean (±SE) rates of responding before injection of water, C, or B. "Component 2—Posttreatment" refers to mean (±SE) rates of responding x hours after the initial injection and 15 min after an injection of water. Flumazenil or flumazenil vehicle was administered immediately following component 2 and tested 15 min later in component 3.

*Significantly different from baseline (Bonferroni, $p < .05$).

pretreated with either 1) water, tested at 2 and 4 h; 2) CDAP 10 mg/kg, tested at 1, 2, and 4 h; or 3) bretazenil 10 mg/kg, tested at 1 h. Flumazenil, 10, 32, and 56 mg/kg, was administered to animals pretreated with 1) water, tested at 1 h; 2) bretazenil, 10, 75, and 100 mg/kg, tested at 1, 2, and 4 h; and 3) CDAP 75 mg/kg, tested at 18 h. Flumazenil, 32, 56, and 100 mg/kg, was administered to animals pretreated with water, CDAP 100 mg/kg, or bretazenil 100 mg/kg and tested at 18 h. Flumazenil, 56 and 100 mg/kg, was administered to animals pretreated with water or bretazenil 75 mg/kg and tested at 18 h. Water, CDAP, and bretazenil were administered in a semirandom fashion, but usually on a test day one third of the animals would receive CDAP, another one third bretazenil, and the remaining one third water or a different dose of CDAP or bretazenil.

Drugs

CDAP HCl was obtained from Sigma Chemical Co. (St. Louis). Flumazenil and bretazenil were graciously supplied by Hoffman-La Roche, Basel, Switzerland (bretazenil) and Nutley, NJ (flumazenil). CDAP was dissolved in saline, and

flumazenil and bretazenil were suspended in distilled water to which 2 drops of Tween 80 per 10 ml had been added. All compounds were mixed immediately prior to testing and all injections were IP. Injection volume was 1 ml/kg, except for the 100-mg/kg doses of CDAP and bretazenil, which were administered in a volume of 2 ml/kg.

Data Analysis

Data were initially analyzed by a two-way Subject × Component repeated-measures analysis of variance utilizing all five components. Results showed that there was no dose-effect function with cumulative doses of flumazenil. This may be because flumazenil has a relatively short half-life (plasma $t_{1/2}$ < 15 min [personal communication from Hoffman-LaRoche]), and the successive doses were not actually cumulative. For this reason, statistical analyses were repeated utilizing only the first three components (i.e., baseline, posttreatment, and the first flumazenil or flumazenil vehicle injection). When there was a significant component effect, post hoc comparisons were done with the Bonferroni test to determine where the significance lay. Significance level was set at $p < .05$.

RESULTS

Table 1 shows the results of flumazenil vehicle and the first dose of flumazenil after pretreatment with water, bretazenil, or CDAP. It can be seen that flumazenil had no effect on rates of responding in water or bretazenil-pretreated animals. In contrast, when rats had received 100 mg/kg CDAP 18 h prior to being tested with flumazenil, there was a significant component effect, $F(2, 26) = 11.59, p = .0003$, and post hoc analysis revealed that this was due to a decrease in responding after 32 mg/kg of flumazenil ($p < .05$). Table 1 also shows that there was a component effect 1 h after pretreatment with 10 mg/kg bretazenil, $F(2, 22) = 3.79, p = .0384$, and 75 mg/kg bretazenil, $F(2, 10) = 9.85, p = .0043$, 4 h after pretreatment with 100 mg/kg bretazenil, $F(2, 14) = 4.48, p = .0314$, and 2 h after pretreatment with 10 mg/kg CDAP, $F(2, 10) = 6.16, p = .0181$. Post hoc analyses revealed that these differences were due to increases in rates of responding in component 2—that is, posttreatment ($p < .05$). These increases in responding were reversed by the lowest dose of flumazenil, and there was no further decrease below baseline rates of responding with successive doses (data not shown).

DISCUSSION

Increased sensitivity to an antagonist following exposure to an agonist has been interpreted as a measure of dependence and an indicator of withdrawal (10,16,33). In the current study, only rats that had received a high (100 mg/kg) dose of CDAP were more sensitive to the effects of flumazenil, and this effect was seen at a relatively high dose of flumazenil (32 mg/kg). It is noteworthy that doses as high as 100 mg/kg bretazenil did not produce dependence, since bretazenil is much more potent than full BDZ agonists in the rat (12). That dose dependency was not seen when cumulative doses of flumazenil were administered 18 h after CDAP 100 mg/kg is probably due to the short plasma half-life (<15 min [personal communication from Hoffman-LaRoche]) and short elimination half-life (approximately 1 h) of flumazenil (7). These data therefore show behavioral evidence of flumazenil's short half-life.

Increased sensitivity to flumazenil was also found in squirrel monkeys one day after a single 10-mg/kg dose of CDAP (28). In that study there was an increase in FI responding one day after CDAP; low doses of flumazenil reversed the increase in responding, while higher doses of flumazenil substantially decreased responding. These results contrast with those of the present study, in that rates of responding were similar to baseline 18 h after a high dose of CDAP, and while the lowest dose of flumazenil significantly decreased responding, higher doses did not further decrease responding.

Increases in FI rates of responding were observed 2 h after 10 mg/kg CDAP, 1 h after 10 and 75 mg/kg bretazenil, and 4 h after 100 mg/kg bretazenil. These increases in responding could be interpreted as either a delayed drug effect or withdrawal. The former seems more likely, however, as the increases in rate of responding were reversed by flumazenil, and successive doses failed to decrease responding to rates that were below baseline. These results are similar to those in the squirrel monkey 1 h after 10 mg/kg CDAP in that rate increases were reversed by flumazenil, but there was no further decrease in rate, whereas when the same monkeys were tested 18 h after the same dose of CDAP, flumazenil not only reversed the rate increases but decreased responding to rates below baseline (28). Similar decreases in operant behavior are seen during abstinence and precipitated opioid withdrawal in the rat (1,29). In precipitated withdrawal studies, animals are typically challenged with the antagonist when the effects of the agonist are no longer apparent, and the decrease in response rate upon administration of the antagonist is believed to be an indicator of withdrawal. A major difference between opioids and benzodiazepines, however, is that acute dependence can be demonstrated in the rat with very low (less than 10 mg/kg) doses of opioids, whereas in the current study precipitated withdrawal was only seen with the highest (100 mg/kg) dose of CDAP.

The finding that bretazenil apparently did not produce physical dependence, as evidenced by a lack of precipitated withdrawal, is in accord with what others have reported (18, 23). In those studies, animals were treated chronically with bretazenil and were then observed during abstinence for signs of withdrawal such as weight loss, seizures, etc. In the present study, animals were not specifically observed for physical signs of withdrawal, but no overt signs of withdrawal from either CDAP or bretazenil were noticed, including weight loss. Others (5) have reported a clearly defined withdrawal syndrome when flumazenil was administered 4 h after a single oral dose of 75 mg/kg CDAP, a time when animals were maximally sedated. In the present study, when animals were tested with a water injection 4 h after 75 mg/kg CDAP (IP) they were unable to respond in the operant chamber and were therefore not challenged with flumazenil. Thus, the possibility exists that overt physical signs of withdrawal might have been observed at that CDAP dose and time interval had flumazenil been administered.

In the present study, despite the absence of overt signs of withdrawal, disruptions in behavior were observed after single high doses of CDAP in both the precipitated and abstinence withdrawal studies. These findings indicate that schedule-controlled behavior can be used as another measure for detecting BDZ withdrawal, and they extend and support the findings of others who have used schedule-controlled behavior to study withdrawal from a number of drugs of abuse (3,6,11,14,27, 28,32).

ACKNOWLEDGEMENTS

Supported by DA 06637. The author is indebted to Prof. W. Haefely of Hoffmann-LaRoche (Basel, Switzerland) and Dr. Peter Sorter, Hoffmann-LaRoche (Nutley, NJ) for the generous gifts of bretazenil and flumazenil, respectively.

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