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Quantitative pharmacological analyses of the interaction between flumazenil and midazolam in monkeys discriminating midazolam: determination of the functional half life of flumazenil

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Abstract

The duration of action of a drug is commonly estimated using plasma concentration, which is not always practical to obtain or an accurate estimate of functional half life. For example, flumazenil is used clinically to reverse the effects of benzodiazepines like midazolam; however, its elimination can be altered by other drugs, including some benzodiazepines, thereby altering its half life. This study used Schild analyses to characterize antagonism of midazolam by flumazenil and determine the functional half life of flumazenil. Four monkeys discriminated 0.178 mg/kg midazolam while responding under a fixed-ratio 10 schedule of stimulus-shock termination; flumazenil was given at various times before determination of a midazolam dose-effect curve. There was a time-related decrease in the magnitude of shift of the midazolam dose-effect curve as the interval between flumazenil and midazolam increased. The potency of flumazenil, estimated by apparent pA_2 values (95% CI), was 7.30 (7.12, 7.49), 7.17 (7.03, 7.31), 6.91 (6.72, 7.10) and 6.80 (6.67, 6.92) at 15, 30, 60 and 120 min after flumazenil administration, respectively. The functional half life of flumazenil, derived from potency estimates, was 57 ± 13 min. Thus, increasing the interval between flumazenil and midazolam causes orderly decreases in flumazenil potency; however, across a broad range of conditions, the qualitative nature of the interaction does not change, as indicated by slopes of Schild plots at all time points that are not different from unity. Differences in potency of flumazenil are therefore due to elimination of flumazenil and not due to pharmacodynamic changes over time.

Keywords

midazolam; flumazenil; benzodiazepine; drug discrimination; rhesus monkey

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1. Introduction

Benzodiazepines positively modulate γ -aminobutyric acid (GABA) through distinct binding sites on GABA_A receptors, and there is little variation among benzodiazepines in pharmacodynamic factors, such as selectivity and efficacy. Consequently, the choice of a particular benzodiazepine for use in the clinic or the laboratory is based primarily on pharmacokinetic factors, and one such factor that varies markedly among benzodiazepines is duration of action. Other drugs act at these binding sites with little or no positive efficacy and can competitively antagonize the behavioral effects of benzodiazepines, thereby providing an important clinical advantage. Only one drug, flumazenil, is currently approved to reverse the effects of benzodiazepines; however, its short duration of action can lead to the reemergence of effects of long-acting benzodiazepines as flumazenil is eliminated (Morse and Bamias, 2010).

Duration of action is also important in research largely because it impacts potency. For antagonists like flumazenil, magnitude of shift of agonist dose-effect curves (e.g., Schild analysis) is used to estimate potency. Differences in antagonist potency (e.g., pA₂) across conditions can indicate the involvement of different subtypes of receptors; however, in vivo, potency also varies according to the interval between administration of the antagonist and agonist. Consequently, while potency differences might indicate multiple receptor mechanisms, pharmacokinetics must also be considered when interpreting these data.

The half life of drug in plasma is often used to estimate duration of action, and the plasma half life of flumazenil is between 60 and 90 min in humans (Bonfiglio et al., 1996; Zhi et al., 1994). While the ability of flumazenil to reverse the effects of benzodiazepines is related to its plasma concentration (Zhi et al., 1994), the potency of flumazenil is not always accurately predicted by plasma concentration. For example, after systemic administration in rats, the concentration of flumazenil is much greater in brain than in blood, and plasma concentration would not predict effects that are centrally mediated (Lister et al., 1984). In addition, pharmacokinetic interactions between flumazenil and some benzodiazepines can alter plasma concentration of flumazenil (Lister et al., 1984) or of the benzodiazepine (e.g., midazolam; Bonfiglio et al., 1996). Together with the fact that it is not always possible to obtain blood or brain tissue, these results underscore the importance of knowing functional half life (i.e., time for the potency of a drug to decrease by half) for drugs, including flumazenil.

In the current study, the functional half life of flumazenil to antagonize a benzodiazepine was determined in monkeys discriminating midazolam. Schild analyses were used to characterize the antagonism as the interval between flumazenil and midazolam increased. Previously published data show that, when flumazenil is given 15 min before midazolam, slopes of Schild plots are not different from unity, indicating simple, competitive and reversible interactions with pA₂ values that range from 7.41 to 7.69 (Lelas et al., 2000; Gerak and France, 2012). This study examined whether the pharmacodynamics of flumazenil changed with longer intervals and estimated the functional half life of flumazenil (Tallarida et al., 1978).

2. Materials and methods

2.1. Subjects

One male and four female adult rhesus monkeys (subjects HE, JA, KI, LI and NI) were individually housed in a temperature-controlled room under a 14-h light and 10-h dark cycle. Monkeys weighed between 7.6 and 9.3 kg and received primate chow (Harlan Teklad, High Protein Monkey Diet, Madison, WI, USA), fruit, and peanuts daily. The female monkeys (subjects HE, JA, LI and NI) had a history of discriminating midazolam from vehicle while responding under a schedule of stimulus-shock termination (Gerak and France, 2011; Zanettini et al., 2013). The male monkey (subject KI) discriminated midazolam from vehicle under a schedule of food presentation immediately before this experiment (Bai et al., 2011). All the animals used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio, Texas, USA, and the 2013 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Science, National Research Council, National Academy of Sciences).

2.2. Apparatus

Experimental sessions were conducted in sound-attenuating, ventilated chambers equipped with two stimulus lights and two levers. Monkeys were seated in primate chairs (Primate Products, Miami, FL) and their feet were placed in shoes attached to the front of the chairs. Shock (250 ms, 3mA) could be delivered to brass electrodes in the shoes. An interface connected the chamber to a computer which controlled experimental contingencies and collected data (MED-PC IV, Med Associates Inc., East Fairfield, VT, USA).

2.3. Procedure

At the beginning of the study, monkeys discriminated 0.178 mg/kg midazolam from vehicle while responding under a fixed-ratio 10 schedule of stimulus-shock termination (Gerak and France, 2012; Zanettini et al., 2013). Over the course of the experiment, 0.178 mg/kg midazolam started to decrease response rates in one monkey (subject NI), and the training dose was changed to 0.133 mg/kg for that monkey; midazolam dose-effect curves were not significantly altered by the change in training dose. Experimental sessions comprised a minimum of 2 and a maximum of 8 cycles with each cycle lasting 15 min. Cycles started with 10-min timeout periods during which stimulus lights were off and responding had no programmed consequence followed by 5-min response periods during which red stimulus lights were illuminated and shock was scheduled to occur every 15 s. Responding 10 consecutive times on the lever designated correct by an injection given at the beginning of the cycle extinguished stimulus lights and postponed the shock schedule for 30 s. Response periods ended after 5 min or delivery of 4 shocks, whichever occurred first.

Injections were given during the first minute of each cycle. For training sessions, one lever was designated correct following injection of the training dose of midazolam and the other lever was designated correct following administration of saline. The cycle in which midazolam was administered was preceded by 0–6 cycles, during which saline and sham injections were given, and followed by one cycle, during which a sham injection was given.

For cycles that preceded administration of midazolam, responding on the saline lever extinguished lights and postponed shock, and for the last two cycles of the session, responding on the midazolam lever extinguished lights and postponed shock. When only saline and sham injections were given during training sessions, responding on the saline lever extinguished lights and postponed shock for all cycles. Stimulus control was considered adequate for testing when responses emitted on the correct lever were 80% of the total responses for the cycle and when no more than 9 responses (i.e., <one fixed ratio) were emitted on the incorrect lever prior completion of first fixed ratio. Before the first test session was conducted under these conditions, monkeys satisfied the criteria for 5 consecutive or 6 of 7 training sessions, which occurred immediately before these studies for subject KI and previously for the other subjects (Zanettini et al., 2013). Thereafter, stimulus control was considered adequate for testing when the criteria were satisfied during two consecutive training sessions.

Test sessions were identical to training sessions with the exception that 10 consecutive responses on either lever extinguished lights and postponed shock. Midazolam dose-effect curves were determined by administering increasing doses across cycles. Saline was given at the beginning of the first cycle and midazolam was given during subsequent cycles with the dose increasing in $\frac{1}{4}$ log unit increments for each cycle (Gerak and France, 2011; Zanettini et al., 2013). On separate occasions, monkeys received an injection of flumazenil (0.01–1 mg/kg) or its vehicle 15, 30, 60, or 120 min before determination of a midazolam dose-effect curve. The initial dose of midazolam for each dose-effect determination varied, depending on the dose of flumazenil and the interval between its administration and the start of the session, so that the entire dose-effect curve could be obtained within 120 min. For all combinations of flumazenil and midazolam, the mean number of doses of midazolam ranged from 5 to 7. Each monkey was tested under all conditions.

2.4. Drugs

Midazolam hydrochloride (Hospira Inc, Lake Forest, IL) was purchased as a commercially prepared solution and diluted with 0.9% saline. Flumazenil (Enzo Life Sciences, Farmingdale, NY) was dissolved in a vehicle of 10% ethanol, 40% propylene glycol, and 50% sterile water. Drugs were administered s.c. in the back in a volume ranging from 0.2 to 1.2 ml. Injection volumes varied due to the weights of monkeys, the limited solubility of flumazenil, and the concentration of midazolam that was available commercially.

2.5. Data analyses

Control response rates for each monkey were calculated by averaging rates across 5 training sessions to obtain a mean rate for each monkey; rates were then averaged across subjects to obtain the group control response rate and standard error of the mean. When rate for an individual subject was less than 10% of its control rate during a cycle, discrimination data would not be included in the analyses for that cycle. The percentage of total responses emitted on the drug lever was plotted as function of midazolam dose. For each monkey, data on the linear portion of the dose-effect curve were used to fit straight lines, which included the largest dose that produced 20% responding on the midazolam lever, the smallest dose that produced 80% responding on the midazolam lever, and all doses in between. Slopes

were compared with an F-ratio test ($\alpha=0.05$). When slopes were not significantly different, the common slope was used to determine intercepts, and from those values, the dose of midazolam needed to produce 50% responding on the midazolam lever (ED_{50}) was calculated for each monkey. Potency ratios were determined for individuals by dividing ED_{50} values of midazolam obtained in the presence of flumazenil by those obtained when vehicle was administered at the same time before the session. ED_{50} values and potency ratios were averaged across subjects and confidence intervals were calculated.

Schild plots were constructed for each interval between flumazenil and midazolam by plotting the logarithm of the potency ratio minus one as a function of the negative logarithm of the flumazenil dose expressed in moles per kilogram (Arunlakshana and Schild, 1959). Straight lines were fitted to each Schild plot using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) and the following equation: $\log(\text{potency ratio}-1) = -\log(\text{molar dose of flumazenil}) \times \text{slope} + \text{intercept}$. Regression lines were compared across pretreatment times using an F-ratio test. When the F-ratio test indicated that the slope was not significantly different from unity, then the slope was constrained to -1 . Apparent pA_2 values were then obtained from the regression lines.

The apparent half life of flumazenil was determined using the method of Tallarida et al. (1978). First, for each dose of flumazenil, the logarithm of the potency ratio minus 1 was plotted as a function of pretreatment time. Slopes of regression lines that were fit to these data were compared using an F-ratio test, and when there was no significant difference across doses, a common slope was used. The regression line is described by the following equation: $\log(\text{potency ratio}-1) = (-\beta \log[e])(\text{pretreatment time}) - \log(\text{flumazenil dose}) + \log(K_b)$ where β is the rate of elimination of flumazenil and K_b is its dissociation constant. From that equation, $\text{slope} = -\beta \log[e]$; thus, the slope of the regression line was used to calculate the rate of elimination, and the apparent half life of flumazenil was then derived using the formula: $t_{1/2} = \ln(2)/\beta$. The standard error of the half life of flumazenil was determined from the error of the best-fit slope by applying the formula of the propagation of the errors: $\text{standard error of } t_{1/2} = (e)(\text{standard error of slope})\log(e)/\text{slope}^2$.

3. Results

The group mean response rate (± 1 S.E.M.) was 2.83 ± 0.54 responses/s during sessions in which only vehicle and sham injections were given and 2.20 ± 0.25 responses/s during sessions in which the training dose of midazolam was administered. Midazolam dose dependently increased responding on the midazolam lever with ED_{50} values (± 1 S.E.M.) of 0.08 ± 0.02 , 0.08 ± 0.01 , 0.11 ± 0.03 , and 0.06 ± 0.01 mg/kg when vehicle was administered 15, 30, 60, and 120 min before midazolam, respectively (circles, Figure 1). Flumazenil dose and time dependently antagonized the discriminative stimulus effects of midazolam, shifting the midazolam dose-effect curve further rightward with increasing doses of flumazenil or decreasing intervals between flumazenil and midazolam. For example, when given 120 min before midazolam, 0.1, 0.32 and 1 mg/kg flumazenil shifted the midazolam dose-effect curve 2.8 ± 0.4 , 9.1 ± 1.5 , and 27.1 ± 4.5 fold rightward, respectively (bottom panel, Figure 1). Similarly, the magnitude of shift of midazolam dose-effect curves produced by a single dose of flumazenil decreased as the interval between flumazenil and midazolam increased;

0.32 mg/kg flumazenil shifted the midazolam dose-effect curve 17.2 ± 3.1 , 10.5 ± 3.5 , and 9.1 ± 1.5 fold rightward when the time after flumazenil was 30, 60, and 120 min, respectively (open diamonds, Figure 1). Response rates in individual monkeys were never decreased to less than 1.30 responses/s by midazolam, flumazenil or any combination of the two drugs (data not shown).

Schild analyses were used to quantify antagonism of midazolam by flumazenil. First, Schild plots were constructed for flumazenil at each interval (Figure 2), which yielded slopes that were not significantly different from unity (15 min: $F_{1,13}=0.04$; 30 min: $F_{1,13}=0.12$; 60 min: $F_{1,12}=0.13$; 120 min: $F_{1,13}=2.12$). The potency of flumazenil was greatest when the interval between flumazenil and midazolam was 15 min; at that time and with slopes constrained to unity, the apparent pA_2 value (95% confidence interval) was 7.30 (7.12, 7.49), and the value decreased in a time-dependent manner to 6.80 (6.67, 6.92) at the longest interval studied (120 min; $F_{3,55}=9.10$, $P<0.01$; Figure 2; Table 1). In addition, shifts in the midazolam dose-effect curve produced by flumazenil were plotted as a function of the interval between flumazenil and midazolam (Figure 3); regression lines were constructed for each dose of flumazenil and their slopes did not differ significantly (slope = -0.005 ± 0.001 ; $F_{2,4}=1.99$), indicating that the data were best fit by the simpler model with a common slope. That common slope, which was significantly different from zero, was used to derive the rate of elimination of flumazenil, which was estimated to be $0.01 \pm 0.002 \text{ h}^{-1}$, yielding an apparent half life of $57 \pm 13 \text{ min}$.

4. Discussion

Flumazenil is used clinically to reverse the behavioral effects of benzodiazepines; however, its duration of action is shorter than many benzodiazepines. When flumazenil is eliminated before the benzodiazepine, unwanted effects can reemerge. Because the plasma concentration of flumazenil might not always predict its functional half life due to differences in rate of elimination from plasma and brain or to pharmacokinetic interactions between flumazenil and benzodiazepines or other drugs (Bonfiglio et al., 1996; Lister et al., 1984), knowing the functional half life of flumazenil is important both when it is used clinically and in order to understand its pharmacodynamics. In this study, the potency of flumazenil to antagonize the discriminative stimulus effects of midazolam was determined by using Schild analyses to estimate the apparent affinity of flumazenil and obtain its functional half life, which was estimated to be $57 \pm 13 \text{ min}$. A similar functional half life of flumazenil would be expected regardless of the particular benzodiazepine used or effect studied because the ability of flumazenil to antagonize benzodiazepines does not differ across benzodiazepines or their effects.

In addition to predicting duration of action of flumazenil, these data provide important information about the pharmacodynamics of flumazenil and benzodiazepines and their interaction at benzodiazepine binding sites on GABA_A receptors. Schild analyses are valuable for estimating affinity of antagonists as well as understanding interactions between drugs and receptors. Although they have been used most often to characterize interactions of drugs acting at G-protein coupled receptors, they are also useful in understanding interactions between drugs acting at modulatory sites on ion channels. For example, when

flumazenil antagonizes the discriminative stimulus effects of midazolam in monkeys, slopes of Schild plots do not deviate from unity (Lelas et al., 1999, 2000; Gerak and France, 2012), indicating that assumptions of these analyses, simple, competitive and reversible interactions, are not violated despite the fact that these are not G-protein coupled receptors. Another important assumption of Schild analyses is that the concentration of antagonist at receptors is at equilibrium, and violations can result in slopes of Schild plots that deviate from unity. While the concentration of antagonist at receptors is not known in vivo, that assumption does not appear to be violated for some antagonists acting at G-protein coupled receptor because slopes of Schild plots do not deviate from unity as time since antagonist administration increases (Gerak and France, 2007). Similarly, increasing the interval between administration of flumazenil and determination of the midazolam dose-effect curve did not alter slopes of Schild plots, suggesting that assumptions, including antagonist equilibrium, are not violated and that this approach is appropriate for characterizing interactions at allosteric sites on ion channels.

Schild analyses also provide quantitative estimates of potency of antagonists, which are routinely compared across various experimental conditions; differences in the potency (pA_2) of an antagonist to attenuate the effects of agonists can reflect pharmacodynamics differences, such as mediation by distinct receptor subtypes. The current study demonstrates that even modest differences in experimental conditions, such as small increases in the interval between administration of antagonist and agonist, affect antagonist potency. These differences are due to the pharmacokinetic properties of the antagonist and not to mediation by different mechanisms, emphasizing the importance of comparing the potency of antagonists under experimental conditions that are as similar as possible before concluding that potency differences are due to pharmacodynamic differences. The current study provides a range of conditions with quantitative estimates of the potency of flumazenil against which other data can be compared, and when differences in potency are detected under similar conditions, then the receptors mediating these effects likely differ. Thus, while differences in the potency of antagonists to block the effects of agonists can reflect contributions of multiple receptors in mediating the effect, variations in potency in the current study are entirely due to differences in the temporal conditions, thereby underscoring the fact that differences in antagonist potency should be interpreted cautiously when experimental conditions are not identical.

The duration of action of a drug is important both in the clinic and in research. When flumazenil is used clinically to reverse the effects of benzodiazepines, it is preferable that the effects of flumazenil outlast those of the benzodiazepine. Because the duration of action of flumazenil can be altered by benzodiazepines (Bonfiglio et al., 1996; Lister et al., 1984), the functional half life is as important as its half life in plasma. Results of the current study indicate a functional half life of less than one hour in monkeys, which is similar to the plasma half life of flumazenil obtained in humans (Bonfiglio et al., 1996; Zhi et al., 1994). It is not always possible to obtain both the functional half life and the plasma half life; however, these results suggest that the half life of flumazenil determined by one of these methods is reasonably predictive of its half life determined by the other method. Moreover, the current study indicates that the half life of flumazenil is not substantially extended by midazolam and suggests that plasma half life obtained in otherwise untreated humans

predicts functional half life of flumazenil in the presence of midazolam. In research, antagonism of benzodiazepines by flumazenil can be used to identify mechanism of action and characterize the interaction between drugs and receptors. Although this approach has not been used extensively to characterize interactions between drugs acting at an allosteric modulatory site on an ion channel, these data demonstrate that this strategy can be used for these interactions as well. Moreover, small variations in pretreatment time significantly altered the potency (pA_2) of flumazenil, underscoring the importance of fully characterizing drug combinations across a wide range of doses and temporal conditions and comparing results under similar conditions before concluding that potency differences are due to differences in mechanism of action.

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Figure 1.

Midazolam dose-effect curves determined 15, 30, 60 and 120 min after administration of vehicle and different doses of flumazenil. Abscissae: dose in milligrams per kilogram body weight. Ordinates: mean percentage of total responding on the midazolam lever (± 1 S.E.M.).

Figure 2. Schild plots for flumazenil given at different times before determination of midazolam dose-effect curves. Abscissa, negative log of the molar dose of flumazenil. Ordinate, log (dose ratio-1).

Figure 3. Time-dependent shifts of the midazolam dose-effect curve produced by different doses of flumazenil. Abscissa, flumazenil pretreatment time (min). Ordinate, log (dose ratio-1).

Table 1

Slopes of Schild plots and apparent pA_2 values (95% confidence interval) of flumazenil when administered at different times before determination of a midazolam dose-effect curve. Apparent pA_2 values were determined when slope was not constrained and when it was constrained to unity (-1).

Time (min)	Slope	pA_2 (unconstrained)	pA_2 (constrained)
15	$-1.04 (-1.54, -0.55)$	7.29 (7.19, 7.55)	7.30 (7.10, 7.49)
30	$-0.94 (-1.29, -0.58)$	7.23 (7.03, 7.67)	7.18 (7.04, 7.32)
60	$-0.98 (-1.43, -0.52)$	6.94 (6.79, 7.36)	6.93 (6.76, 7.10)
120	$-1.20 (-1.50, -0.90)$	6.66 (6.55, 6.88)	6.79 (6.69, 6.92)