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Effect of 7-day exposure to midazolam on electroencephalogram pharmacodynamics in rats: a model to study multiple pharmacokinetic–pharmacodynamic relationships in individual animals

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Abstract

The objective of this study was to determine the concentration–electroencephalogram (EEG) relationships for midazolam, a full-agonist benzodiazepine ligand, on multiple occasions in individual rats, and to examine the effect of chronic midazolam exposure on that relationship. Rats were chronically instrumented with venous and arterial cannulas, and cortical EEG electrodes. The rats received either: 7 days of midazolam 10 mg kg^{-1} intravenously once a day (midazolam group); or midazolam on days 1 and 7 and vehicle on days 2–6 (vehicle group). Concentration–effect relationships were determined on days 1, 4 and 7 from multiple blood and EEG samples before and after the administration of the midazolam dose. The concentration–EEG effect relationships were consistent with a sigmoidal E_{max} (maximal effect) model. No differences in pharmacokinetic or pharmacodynamic parameters were found between day 1 and day 7 in either group. However, in the midazolam group, both the fraction unbound of midazolam in serum and the EC50 (concentration at half-maximal effect) for free midazolam increased from days 1–7 by 35±3% and 54±25%, respectively (means±s.d., $P < 0.05$). This may be related to decreased serum albumin levels in the midazolam group (−19±5%, $P < 0.05$) which, in turn, could be explained by the sedation associated with daily midazolam treatment. We concluded that concentration–EEG effect relationships can be studied on multiple occasions in individual animals, reducing animal use and variability. A modest degree of tolerance to midazolam was found with this paradigm, the effect only being evident after correction for the fraction unbound of midazolam.

Introduction

Benzodiazepine agonists are widely used clinically for their sedative-hypnotic, anticonvulsant, and anxiolytic properties (Hollister et al 1993; Shader & Greenblatt 1993). These compounds enhance the effect of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in mammalian CNS, by binding to a specific site on the $GABA_A$ receptor. Over the years, concentration–effect relationships have been established for a number of benzodiazepine agonist effects in experimental animals and humans (Laurijssens & Greenblatt 1996). Some experimental models allow study of the complete concentration–effect relationship in individual subjects. One such model, the effect of benzodiazepines on the electroencephalogram (EEG) in a cannulated rat, depicts their action at the benzodiazepine site on the $GABA_A$ receptor. This model reflects the spectrum of

intrinsic activities (full, partial, and inverse agonism as well as antagonism) at the benzodiazepine receptor, as well as the ranking of the anticonvulsant potency and receptor affinity of different agonists (Mandema et al 1991a, b, 1992a, b).

Factors influencing the pharmacodynamic response to benzodiazepines, as well as the regulation of the receptor, include age, disease, concurrent medications and extended exposure (Miller 1991; Laurijssens & Greenblatt 1996). Understanding of the phenomenon of benzodiazepine tolerance is complicated by the use of different benzodiazepine ligands, exposure and exposure patterns, and effect measures in different species and in-vitro systems. Tolerance to the sedative and anticonvulsant effects of benzodiazepines is most often reported clinically. Tolerance to the hypnotic effects is less evident and tolerance to anxiolytic efficacy is minimal (Woods et al 1992; Hutchinson et al 1996).

This study evaluated the concentration–EEG effect relationships on multiple occasions in individual rats for the benzodiazepine midazolam, and assessed the effect of extended midazolam exposure on that relationship. Midazolam has been previously used in such models (Mandema et al 1991b) and has favourable characteristics including water solubility, short elimination half-life, and, in the rat, minimal production of active metabolites.

Materials and Methods

Drugs

Diazepam and midazolam were kindly provided by Hoffmann-LaRoche Inc., Nutley, NJ. U-31485 (a triazolobenzodiazepine analogue) was provided by the Upjohn Co., Kalamazoo, MI, USA. The vehicle for midazolam was 0.9% saline, pH 3.0.

Experimental animals and study design

The study protocol was approved by the Animal Research Committee of Tufts University School of Medicine and New England Medical Center, Boston, MA. Principles of laboratory animal care were followed.

Male Sprague Dawley rats (250–330 g) (Taconic, Germantown, NY) were housed individually, beginning 1 week before surgery, in a dedicated room in the animal facility, four rats at any given time. All procedures and experiments were performed in this room. The room was controlled for temperature (74–80°F), light and sound, and the computer monitor was active during the entire period. Rats were allowed free access to food and water, except on days on which EEG and blood samples were taken after midazolam administration.

Upon arrival, the rats were assigned to two groups: the midazolam group received once-daily injections (between 0900 and 1200 h) of midazolam 10 mg kg^{-1} intravenously for 7 days; the vehicle group received midazolam 10 mg kg^{-1} intravenously on day 1 and day 7, and vehicle on days 2–6. On any given experimental day, two rats could be tested—one from each group. For every new pair of rats, the equipment was alternated between the groups.

Surgery was performed after 1 week of adaptation to the housing conditions. On day 6 after surgery, an adaptation trial was performed using vehicle injection, without blood sampling. Pilot experiments demonstrated no effect of sampling on the EEG (data not shown). Two days later, the experimental treatments started. On days 1, 4 and 7, the rats were connected to the instrumentation, and blood and EEG samples were taken after the 10 mg kg^{-1} dose of midazolam. Rats in the vehicle group received vehicle on day 4 and blood samples were taken as in the midazolam group. During the study, rats were weighed and monitored for obvious signs of bad health, such as stress excretions around the eye, nose and nails, and piloerection. After the study, autopsies were performed to verify cannula-tip location and condition as well as to visually inspect the kidney surface. The kidney surface was examined for signs of infarction since carotid artery cannulas have been associated with kidney infarcts (Cocchetto & Bjornsson 1983).

Deviations from the experimental design were as follows. One rat from the midazolam group had the electrodes and the cannulas implanted in two separate surgeries on two different days. In addition, one rat in the vehicle group had surgery 14 days before treatment, and a second rat had the cannulas reversed (arterial for administration and venous for sampling) since the arterial cannula did flush but could not draw. In a previous study (Kotegawa et al 1998) we demonstrated that following a single intraperitoneal bolus dose of midazolam 10 mg kg^{-1}, the plasma concentrations were identical whether sampled from the jugular vein cannula or the carotid artery cannula.

Surgery

Procedures were performed under anaesthesia with ketamine–xylazine, 10:1. The initial dose was 30 mg ketamine/3 mg xylazine intraperitoneally. Follow up

doses were 5 mg ketamine/0.5 mg xylazine intravenously. The rats were implanted with two cannulas, venous and arterial, and 5 cortical surface electrodes. Both cannulas were made from silicone rubber tubing (i.d. 0.51 mm, o.d. 0.94 mm; Dow Corning, Auburn, MI). The arterial cannula used a smaller-diameter tip (i.d. 0.31 mm, o.d. 0.64 mm) (see also Kotegawa et al 1998). Before implantation, the cannulas and the electrodes were sterilized by γ-irradiation. The venous cannula was inserted in the right jugular vein and the tip was positioned in the superior vena cava. The arterial cannula was inserted in the left carotid artery and its tip was advanced slowly to its position in the descending aorta, approximately 1 cm beyond the aorta/carotid junction. Electrodes consisted of a stainless-steel machine screw (no. 0-080 $\times$ 1/8″, Small Parts Inc., Logansport, IN) connected to a male amphenol pin (FHC, Brunswick, ME) by 1 cm of insulated wire. The electrodes were screwed into the skull at the following positions (Mandema et al 1991b): 11 mm anterior, $\pm$2.5 mm lateral (F_r and F_l) and 3 mm anterior, $\pm$3.5 mm lateral (C_r and C_l) from lambda, and on lambda.

Both cannulas were filled with heparinized saline (20 IU mL^{-1}) plugged with small pieces of fitting metal rod, and tunnelled under the skin, between the ear and the shoulder to the skull. Cannulas and electrodes were fixed and insulated on the skull with cranioplastic cement (Plastics One, Ranaoke, VA). The cannulas were protected by the top 1.5 cm of a PS test tube (Falcon 13–100 mm; Becton Dickinson, San Jose, CA) with screw cap, which was also embedded in the cement. A plug constructed from the female amphenol pins protected the electrodes. After surgery, the rats received 2 mL of saline and 5.5 mg kg^{-1} enrofloxacin (Baytril; Bayer, Shawnee, KA) subcutaneously.

Beginning the day after surgery, cannulas were checked daily for patency and flushed with 200 μL heparinized saline. If possible, a small volume of blood was drawn and discarded before flushing.

Pharmacokinetic–pharmacodynamic experiment

The pharmacokinetic–pharmacodynamic procedures were adapted from Mandema et al (1991b) and have been described briefly previously (Kotegawa et al 1999). On the day of the experiment, the rats were weighed, cannulas were checked, and electrode impedances measured. The rats were then connected to a PE50 tube (PE50, Becton Dickinson, San Jose, CA) for blood sampling and with a lightweight cable (Cooner Wire, CA) to the EEG instrumentation (bipolar mode: F_r-C_r and F_l-C_l with lambda as the reference electrode), consisting of 7P511J Amplifiers (Grass, Quincy, MA) connected to a PC system (A/D board of Data Translation, Marlboro, MA) and EEG software (Rhythm 9.0; Stellate Systems Quebec, Canada). The recording settings were: high-pass filter 1 Hz, low-pass filter 100 Hz, notch filter (60 Hz), and a sampling rate of 256 Hz. During the entire experiment, the rats were freely moving in their home cage, which was placed on a gel shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The shaker served as vigilance control; during the sampling of the EEG (1-min episodes), the shaker produces gentle horizontal rotary motion (100 rev min^{-1}). Vigilance state has been shown to affect the amplitude in the β-frequency band (e.g. our observations and Schwarz et al 1982). After 2 baseline EEG samples and 1 blood sample were taken, the rats received 10 mg kg^{-1} midazolam by intravenous bolus. For the next 4–6 h, multiple EEG samples (1 min each) and blood samples (100–200 μL) were collected. The last 3 samples were pooled for measuring serum protein binding (total volume 1 mL). Each blood sample volume was replaced with heparinized saline. The blood samples were kept at room temperature. At the end of the day, samples were centrifuged and the serum was stored at -20°C. The EEG samples were visually inspected for artifacts and subsequently fast Fourier transformed (2-s epochs, averaged over 1 min). The EEG was quantified as amplitude (μV) in the following frequency bands: 0.5–2 (delta), 2.5–7 (theta), 7.5–12.5 (alpha) and 13–31 Hz (beta).

Drug analysis

The serum samples were analysed for midazolam using gas chromatography with electron capture detection (Arendt et al 1984). Diazepam (25 ng) and a triazolobenzodiazepine analogue, U-31485 (Greenblatt et al 1981) (50 ng) were added as internal standards, along with 0.5 mL water, to all study samples and to calibration standards, followed by extraction with 2 mL toluene–isoamyl alcohol (98.5:1.5) for 30 s on a vortex mixer. The organic layer was separated, transferred to a 2-mL autosampling vial and evaporated to dryness under reduced pressure. The residue was reconstituted in 225 μL of toluene–isoamyl alcohol–Asolectin (83:14:3) and vortexed. The autosampler was set to deliver 5 μL per injection. The chromatographic column was 6 feet in length, 2 mm internal diameter and packed with 3% OV25 or SP2250 (Supelco, Bellefonte, PA). The oven temperature was 275°C and the carrier gas was 95% argon–5% methane (40–45 mL min^{-1}). The

detection limit for midazolam is 1 ng/tube, equivalent to 10 ng mL^{-1} for a 100-μL sample. The coefficient of variation for identical samples did not exceed 7%. The α- and 4-hydroxy metabolites of midazolam are detected as well by this method, but negligible amounts were measured in the samples. All samples (all experimental days) for a given rat were analysed on the same day using the same calibration standards.

Protein binding

Midazolam protein binding was analysed using equilibrium dialysis. The serum samples (450 μL) were spiked with midazolam to a final concentration of 1 μg mL^{-1}. Previous studies showed the fraction unbound of midazolam to be independent of midazolam concentration over a range of 10–10000 ng mL^{-1} (Moschitto & Greenblatt 1983). The small diameter dialysis tubing (Spectra/Por, 0.1 mL cm^{-1}; MW cut-off 15000; Spectrum, Laguna Hills, CA) was soaked in water for 3–4 h before filling with 200 μL (duplicate) spiked serum, and closed with knots. The filled tubing was submerged in 1.5 mL phosphate buffer pH 7.4 and incubated at 37°C in a shaking water bath for at least 20 h. After incubation, the serum and dialysate were frozen and stored until analysis for midazolam by gas chromatography. The fraction of unbound drug (% Fu) was calculated as $100 \cdot Cu/Ct$, where Cu is the unbound concentration (in dialysate fraction), and Ct is the total concentration (free plus bound) in the serum fraction.

Albumin

Serum albumin concentrations were determined colorimetrically using the bromcresol green method (Doumas & Biggs 1972). Reagent and standards were obtained from Sigma Diagnostics (St Louis, MO). Briefly, 300 μL of Bromcresol Green Reagent was added to 3 μL of the spiked serum samples used for protein binding (see above), and the standards (0–8 g dL^{-1}). UV absorption was read in a 96-well microplate reader (Dynatech Laboratories, Inc., Chantilly, VA) at 570 nm. All samples were measured in triplicate on one plate. Within-run variability was 1–3% (n = 5) as determined with 2 human and 1 rat serum samples.

Data analysis

Effect was analysed as percent of baseline amplitude ($E = 100 \cdot E_t/E_0$) in the beta band (13–31 Hz) of the EEG. Baseline amplitude (E_0) was calculated as the average of two baseline EEG samples. This analysis was performed for both the left hemisphere (F_l-C_l) and the right hemisphere (F_r-C_r). The effects for the right and left hemisphere were very similar and were therefore averaged.

Pharmacokinetics and pharmacodynamics were determined for each individual rat for each experimental day. Serum concentration data were fitted to a linear sum of two exponential terms using standard pharmacokinetic methods. The fitted function was used to interpolate the concentrations at the time of the effect measurement. The concentration–effect data were then fitted to a sigmoidal E_{max} (maximal effect) model:

$$E = 100 + \frac{E_{max} \cdot C^n}{C^n + EC50^n} \quad (1)$$

where E is the pharmacodynamic effect, C is the serum drug concentration and n is the shape factor. An EC50 (concentration at half-maximal effect) based on the unbound midazolam concentrations (ECu50) was calculated by multiplying the EC50 by the % Fu for each experimental day, assuming that the % Fu in a single sample at the end of that experimental day is representative of all samples.

Statistics

SigmaPlot 4.0 was used for all nonlinear regressions and SigmaStat 2.03 was used for all statistical analyses (SSPS Inc., Chicago, IL). The pharmacokinetic and pharmacodynamic parameters were expressed as percent change from the day 1 value of each rat. Statistical comparisons were performed using paired or independent *t*-tests, as appropriate. The study was designed to detect a +100% change in the EC50 (a doubling of the day 1 value) with 80% power and $\alpha = 0.05$. The s.d. was expected to approximate 25% (change from day 1).

Results

Rats

Eight out of 15 rats did not complete the study because of premature death (3), failed arterial cannula (2), very low clearance (CL) of midazolam causing sustained midazolam serum levels (2) or EEG cable failure (1). The rats that were included in the final analysis showed no obvious signs of bad health. The mean body weights of the two groups during the study are shown in Figure 1. Four of the rats (2 in each group) could also be studied on day 14, 7 days after ending the

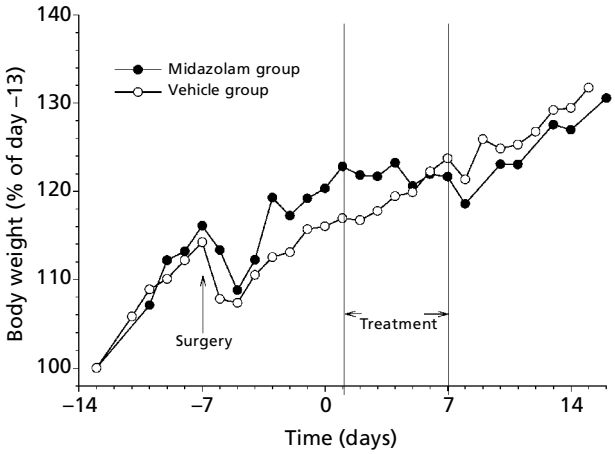

Figure 1 Mean body weight during the study of the midazolam-group and vehicle-group rats. Body weight is normalized for the body weight at arrival (day −13). Up to day 7, n = 4 for the midazolam group and n = 3 for the vehicle group. On other days, n is less, since rats dropped out. Surgery was at day −7 (arrow) and the 7-day treatment period is delineated by the vertical lines.

midazolam or vehicle treatment (results not shown). With one exception, all rats had kidney infarcts.

Pharmacokinetics and pharmacodynamics

The EEG effect and midazolam serum concentrations over time, as well as the concentration–effect relationships after intravenous midazolam on days 1, 4 and 7 of the treatment period are shown for a representative rat from the midazolam group in Figure 2 and a vehicle-group rat in Figure 3. Vehicle injections produced no change in the EEG. Midazolam clearance between days 1 and 7 tended to increase in both midazolam and vehicle groups, but there was no significant difference between groups (Table 1).

The concentration–effect relationships were consistent with a sigmoidal E_{max} model (Figures 2C and 3C), and the determined parameters are summarized in Table 1. When expressed as a percent change from day 1, none of the pharmacodynamic parameters (E_0, E_{max}, EC50, and n) differed significantly from zero change (paired *t*-test) on day 4 or day 7. Also, no significant differences were observed between the two groups on those days (*t*-test).

The free fraction of midazolam in serum significantly increased by 35±3% from day 1 to day 7 in the midazolam group only. Consequently, the ECu50 in that group was significantly increased from day 1 to day 7 by 54±25%, suggesting some degree of pharmacodynamic tolerance. In the same samples in which the free fraction was determined, albumin levels decreased significantly over the treatment period in the midazolam

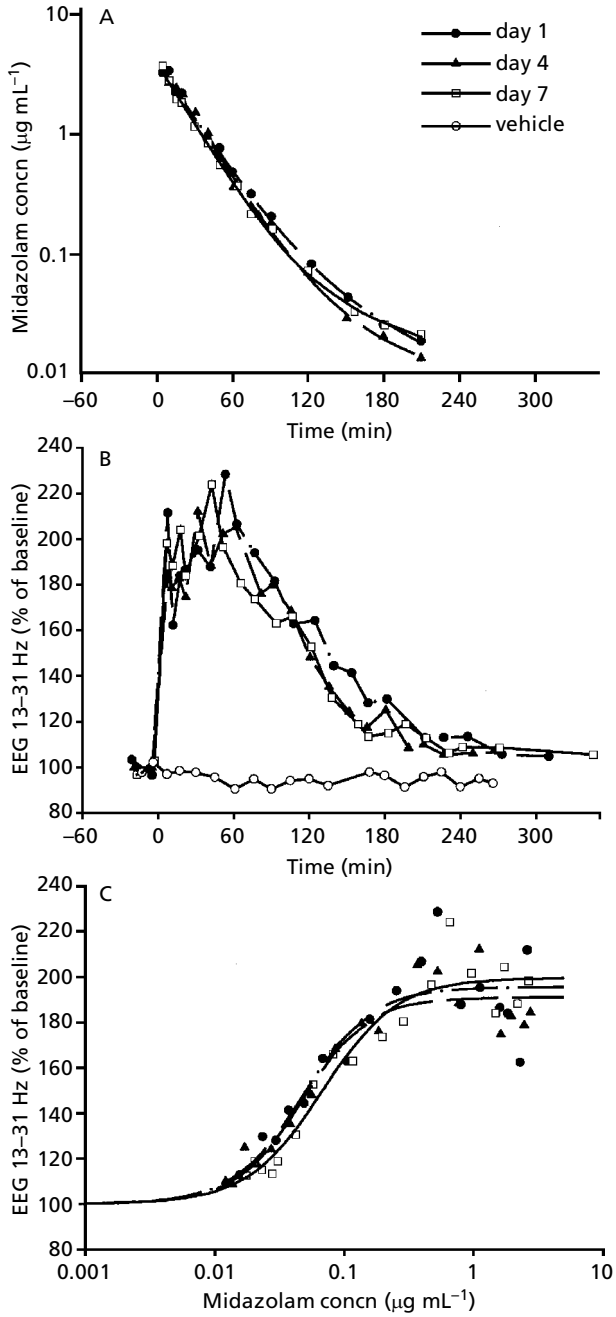

Figure 2 Midazolam serum concentrations (A) and EEG effect over time (B) and their relationship (C) after 10 mg kg^{-1} midazolam intravenously for 1 representative rat of the midazolam group on days 1, 4 and 7 of a 7-day exposure to 10 mg kg^{-1} intravenous midazolam once daily. The vehicle experiment was performed 2 days before the start of the treatment.

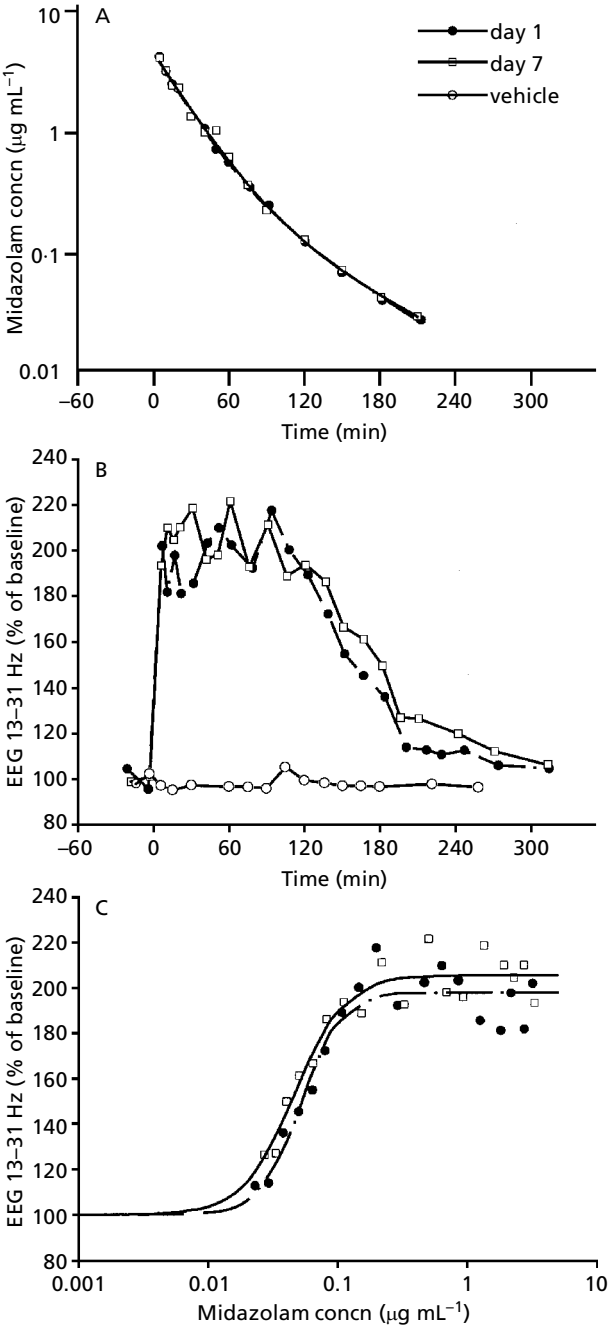

Figure 3 Midazolam serum concentrations (A) and EEG effect over time (B) and their relationship (C) after 10 mg kg^{-1} midazolam intravenously for 1 representative rat of the vehicle group on days 1 and 7, separated by 5 days of intravenous vehicle injections. The vehicle experiment was performed 2 days before the start of the treatment.

group by only 19±5%. Figure 4 shows the effect of treatment on % Fu, albumin, EC50 and ECu50 for all individual rats.

Discussion

This study demonstrates that the relationship between midazolam serum concentration and its effect on the EEG can be studied on multiple occasions in individual animals.

The chronic benzodiazepine paradigm of once daily 10 mg kg^{-1} intravenous midazolam for 7 days did not result in tolerance in the β-band of the EEG, as illustrated by the unchanged concentration–effect relationship based on total (free+bound) serum midazolam levels. However, mild tolerance (50% increase in EC50) was observed when the EC50 was corrected for the fraction of the drug unbound (% Fu). This was observed in all four midazolam-group rats. It should be noted that the % Fu was determined only once for each trial, and this value was assumed to apply to all samples for that trial. In the vehicle group, no changes were observed between the two concentration–effect relationships, separated by one week, with or without the correction for % Fu. The pharmacokinetic (CL, % Fu) and pharmacodynamic (EC50, ECu50, n) parameters for midazolam found in this study are similar to those reported previously (Mandema et al 1991a, b, 1992a).

The EC50 based on the unbound concentration, ECu50, is theoretically more relevant than the uncorrected EC50 (based on total concentration), since it is presumably the unbound midazolam that will equilibrate with the effect site (the CNS) (Arendt et al 1983, 1987). In a study using the original model, the EC50 of midazolam based on CSF concentration was 4.3 ng mL^{-1}, which was similar to the ECu50 of 3.7 ng mL^{-1} (3.6 and 5.4 ng mL^{-1} in our study), and corresponded to the binding K_i for the benzodiazepine receptor determined in-vitro (4.9 ng mL^{-1}) (Mandema et al 1992a).

The increased % Fu observed in the midazolam group, but not in the vehicle group, may be attributed to decreased levels of serum albumin, which are associated with reduced health and chronic cannulation (Cave et al 1995; Rowland & Tozer 1989), although values of % Fu and serum albumin were not directly correlated. In both groups, albumin levels on day 1 were already lower than levels in control rats (4.1±0.3 g dL^{-1}, mean±s.d., n = 3), and levels continued to decline in the midazolam group during the 7 days of chronic treatment. Furthermore, the midazolam group had no net body-weight gain over that period. Thus, the changes in serum albumin and % Fu approximately correspond to the observed changes in body weight. Both groups experienced the same experimental procedures: extensive blood sampling (approximately 3 mL) and 6 h without

Table 1 Summary of the pharmacokinetic and pharmacodynamic parameters of midazolam in rats.

Parameter	Midazolam group (n = 4)					Vehicle group (n = 3)		
				% Change from day 1				% Change from day 1
	Day 1	Day 4	Day 7	Day 4	Day 7	Day 1	Day 7	Day 7
CL (mL min^{-1} kg^{-1})	89±7	95±9	98±3	+7±5	+11±6[b]	89±26	102±25	+17±23
Fu (%)	8.5±4	12±5.3	11.4±5.4	+48±39	+35±3[b,c]	12.3±2.5	11.4±4.1	−7±23
Albumin (g dL^{-1})[a]	3.1±0.1	2.5±0.4	2.6±0.2	−21±12	−19±5[b]	2.9±0.2	2.7±0.1	−7±6
E_0 (μV)	121±9	124±17	125±15	+2±6	+3±6	114±12	108±8	−5±5
E_{max} (% change)	102±9	93±10	87±10	−9±8	−14±14	85±12	80±26	−13±20
EC50 (ng mL^{-1})	39±11	33±13	45±18	−19±13	+15±17	41±24	41±22	+5±16
ECu50 (ng mL^{-1})	3.6±2.4	4.2±2.9	5.8±4.9	+18±34	+54±25[b,c]	5.4±3.6	4.7±3	−3±28
n (shape factor)	1.6±0.4	1.3±0.3	1.5±0.3	−13±21	+8±53	2.2±0.6	2.3±1.1	+15±74

Parameter estimates were determined for each individual rat on each experimental day during either 7 days of once daily 10 mg kg^{-1} intravenous midazolam (midazolam group) or midazolam only on day 1 and 7 with vehicle on days 2–6 (vehicle group). Values are expressed as mean±s.d. for both absolute values and % change from day 1. Statistical analysis was performed only on % change values. [a]n = 3; one rat (midazolam group) was excluded from the means and statistical analyses, because of low day 1 values. Albumin (g dL^{-1}) levels were: day 1, 1.7; day 4, 3.3; and day 7, 2.4. [b]$P < 0.05$,% change from day 1 (paired *t*-test). [c]$P < 0.05$, compared with vehicle group (*t*-test).

food and water on each experimental day, but only the midazolam group received midazolam on days 2–6. Thus, the sedation due to midazolam may have reduced food and water intake. Other studies have shown that chronic cannulation by itself does not dramatically alter body-weight gain (Burt et al 1980; O'Neill & Kaufman 1990; Cave et al 1995).

Factors such as the health of the rats and the patency of drug administration and sampling cannulas limited the duration of study to 7 days. Dose and administration routes (10 mg kg^{-1} i.v. bolus midazolam once a day) were chosen such that they were identical on both the experimental days (when the concentration relationships were determined) and other treatment days. Whether this treatment schedule could induce tolerance to the EEG effect of midazolam in the rat was not certain. Some studies have found tolerance using lower doses within 7 days of treatment (Griffiths & Goudie 1987; King et al 1987; Sanger & Zivkovic 1987), whereas other studies have not, despite substantially higher doses and longer treatment periods (Boisse et al 1990; Vigorito et al 1991; Ramsey-Williams et al 1994). All of these studies, however, evaluated different pharmacological end-points, with midazolam administered either orally or subcutaneously.

Previous studies that examined the effect of chronic benzodiazepine exposure on the EEG did not exclude the possibility of tolerance. Reduction of the spindle duration after a diazepam intravenous bolus was observed on day 3 of chronic administration, and returned to day 1 values at 21 days after discontinuation of treatment. The duration of 20–25-Hz periods (part of the β-band) showed the opposite trend (Mele et al 1984). Vigilance was not controlled in these studies, and the spindles were associated with sedation. Similar effects were found with extended exposure to flunitrazepam, but not clonazepam; extended exposure to an agonist for the peripheral benzodiazepine receptor (PBR) could induce cross-tolerance to diazepam, suggesting a role for the PBR in the observed tolerance (Valerio & Massotti 1988; Ambrosio et al 1992). Midazolam does bind to the PBR, but with only moderate affinity; its intrinsic efficacy is not certain (Bourguignon 1993). Furthermore, tolerance in total power has been seen at week 3 of once daily chlordiazepoxide, but the effect on the β-frequency band was rather small compared with our study (Sala et al 1995). The only human study in alcoholic inpatients did not find any change in the frequency bands after 3 weeks of diazepam (15 mg daily) (Schwarz et al 1982). Since the plasma levels of diazepam and its metabolite increased slightly over that period, tolerance cannot be excluded.

The moderate tolerance observed in our study is not pharmacokinetic but rather pharmacodynamic, reflected in a decrease in potency of midazolam (increase in ECu50). Our study does not elucidate the underlying mechanism of tolerance. Different mechanisms have been proposed, including changes in receptor number and functional uncoupling of the GABA and the benzodiazepine binding sites (Hutchinson et al 1996), and

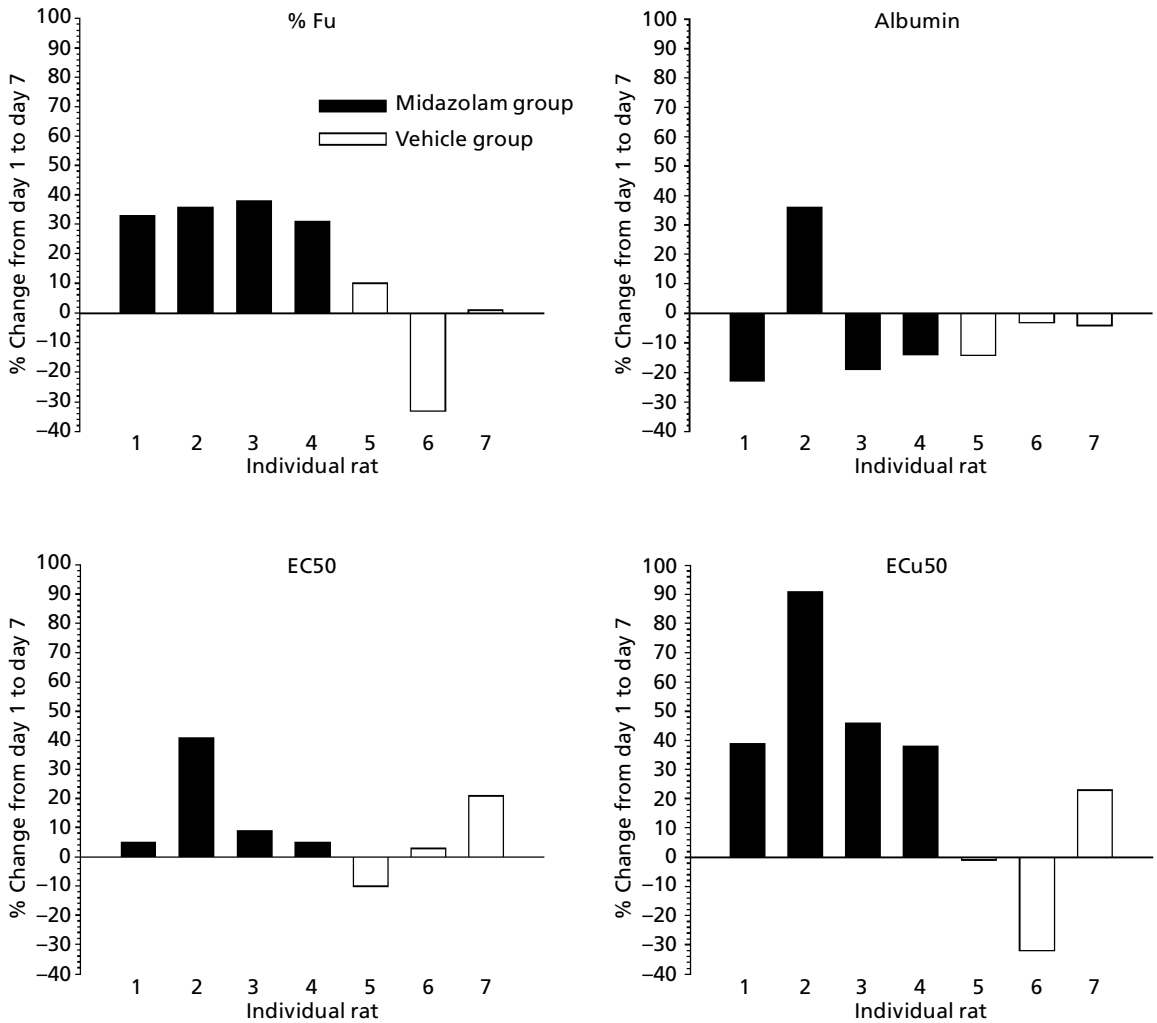

Figure 4 Percent change from day 1 of: the free fraction of midazolam in serum (% Fu); serum albumin; the EC50; and the EC50 corrected for the serum free fraction (ECu50). Values are shown for all individual rats in the midazolam and vehicle groups.

changes in whole neuronal pathways and different receptor systems are also possible. Chronic midazolam-induced changes in $GABA_A$-benzodiazepine receptor functioning are likely to be reflected in the concentration–EEG effect relationship (see Introduction). Thus, our results indicate that changes in receptor function cannot be excluded, and also that these changes appear to be modest in magnitude. Although correlations between EEG β-amplitude and anxiolytic, anticonvulsant and sedative properties of benzodiazepines have been demonstrated (Mandema & Danhof 1992; Laurijssens & Greenblatt 1996), one cannot conclude that tolerance to the EEG effect will parallel tolerance to more clinically relevant effect measures.

Almost all of the rats developed kidney infarcts, and some were found to have an abscess at the carotid artery–aorta junction. The abscesses could be avoided in subsequent studies by immersing the cannula tip in antibiotic before insertion combined with a pre-surgery dose of antibiotic. Kidney infarcts will probably remain a problem with chronic cannulation of the carotid artery, even if careful surgical procedures and maintenance can reduce the size and number of these infarcts (see also Cocchetto & Bjornsson 1983).

In conclusion, it is possible to study full concentration–EEG effect relationships for midazolam on multiple occasions in the same animal. This allows for the study of different modifiers of drug effect within the same animal, such as the chronic treatment demonstrated in this study. The discussed paradigm produced only

a moderate degree of tolerance, but only when the EC50 was corrected for differences in protein binding. Furthermore, this technique allows extensive data to be collected from a single animal, reducing the number of animals used and experimental variability.

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