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Rate of change of blood concentrations is a major determinant of the pharmacodynamics of midazolam in rats

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1 The objective of this investigation was to characterize quantitatively the influence of the rate of increase in blood concentrations on the pharmacodynamics of midazolam in rats. The pharmacodynamics of midazolam were quantified by an integrated pharmacokinetic-pharmacodynamic modelling approach.

2 Using a computer controlled infusion technique, a linear increase in blood concentrations up to 80 ng ml^{-1} was obtained over different time intervals of 1–6 h, resulting in rates of rise of the blood concentrations of respectively, 1.25, 1.00, 0.87, 0.46, 0.34 and 0.20 ng ml^{-1} min^{-1}. In one group of rats the midazolam concentration was immediately brought to 80 ng ml^{-1} and maintained at that level for 4 h. Immediately after the pretreatment an intravenous bolus dose was given to determine the time course of the EEG effect in conjunction with the decline of midazolam concentrations.

3 The increase in β activity (11.5–30 Hz) of the EEG was used as pharmacodynamic endpoint. For each individual animal the relationship between blood concentration and the EEG effect could be described by the sigmoidal E_{max} model. After placebo, the values of the pharmacodynamic parameter estimates were $E_{max} = 82 \pm 5$ μV, $EC_{50,u} = 6.4 \pm 0.8$ ng ml^{-1} and Hill factor $= 1.4 \pm 0.1$. A bell-shaped relationship between the rate of change of midazolam concentration and the value of $EC_{50,u}$ was observed with a maximum of 21 ± 5.0 ng ml^{-1} at a rate of change of 0.46 ng ml^{-1} min^{-1}; lower values of $EC_{50,u}$ were observed at both higher and lower rates.

4 The findings of this study show that the rate of change in plasma concentrations is an important determinant of the pharmacodynamics of midazolam in rats.

Keywords: Pharmacokinetics; electroencephalogram; functional adaptation; computer controlled infusion

Abbreviations: CCIP, computer controlled infusion pump; Cl, clearance; C_t, target concentration; dC/dt, rate of rise in blood concentration; DZP, diazepam; E_0, baseline EEG effect; $EC_{50,u}$, free concentration at half maximal effect; EEG, electro encephalogram; E_{max}, maximum EEG effect; P/B, plasma-to-blood concentration ratio; $t_{½}$, terminal half-life; Vdss, volume of distribution at steady-state

Introduction

Benzodiazepines are well known drugs for their sedative, anxiolytic, anticonvulsant and muscle relaxant effects. Several reports indicate that both acute and chronic functional adaptation to effects of benzodiazepines may occur in man and in animals (Breimer *et al*., 1992; Ellinwood *et al*., 1983; Gallager *et al*., 1985; Haigh, 1988; Smith & Kroboth, 1987). It has been well established that tolerance development to the actions of benzodiazepines is functional in nature (Coasta & Giudotti, 1996). However the understanding of the time course of benzodiazepine-induced tolerance incomplete.

In theory functional tolerance development may be the result of changes at receptor level, at the post receptor level or of changes in homeostatic control mechanisms (Poulos & Cappell, 1991). Much research has been done on the mechanisms involved in tolerance development to the actions of benzodiazepines and several mechanisms have been proposed, such as down-regulation of the benzodiazepine receptors and uncoupling of the benzodiazepine and GABA receptor (Gallager *et al*., 1984; Miller *et al*., 1988). So far however no clear picture has emerged. Regardless of the understanding of the mechanism of functional tolerance development, knowledge of factors that may change the pharmacodynamics *in vivo* is of considerable importance. So far, very limited information is available regarding the factors that determine the rate and extent of functional adaptation to benzodiazepines *in vivo*. It has been suggested that the rate of drug administration may be an important determinant of the clinical effects of benzodiazepines (Ellinwood *et al*., 1985; Greenblatt *et al*., 1977; Grundström *et al*., 1978; Kroboth *et al*., 1993). However, no quantitative information is available on the relationship between the rate of drug administration and the pharmacodynamics of benzodiazepines. This can probably be partly explained by the lack of methods to characterize the pharmacokinetics in small laboratory animals and of methods to control the rate of change of the plasma concentrations *in vivo*. Recently however, several studies on the pharmacokinetics of different bezodiazepines in rats have been reported (Mandema *et al*., 1991a,b; 1992a,b; Hoogerkamp *et al*., 1996). Furthermore computer controlled infusion techniques have been developed for a wide variety of different drugs which allow one to control carefully the plasma concentration. (Alvis *et al*., 1985; Ausems *et al*., 1985; Bührer *et al*., 1987; Martin & Ahuja, 1988; Tackley *et al*., 1989). It has therefore become possible to design infusion regimens and to administer drugs in such a way that it results in a linear increase of the blood concentration at a predefined rate. This is an important advantage over the traditional zero-order infusion techniques, where the blood concentration increases in a non-linear fashion as it reaches steady-state. Application of these innovative computer controlled infusion techniques offers

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therefore a new and unique approach to examine the influence of the rate of change of the blood concentration on the pharmacodynamics. The objective of the present investigation was to characterize influence of the rate of rise of the blood concentration on the pharmacodynamics of benzodiazepines *in vivo*. By application of the recently developed computer controlled infusion technique (Gustafsson *et al*., 1992), the influence of the rate of rise of concentration on the pharmacodynamics of the model drug midazolam was investigated, using quantitative EEG parameters as a pharmacodynamic endpoint.

Methods

Animals and EEG electrode implantation

All experiments were performed in male SPF Wistar rats, weighing 200–250 g (Harlan C.P.B., Zeist, The Netherlands). The rats were housed individually in plastic cages, at a constant temperature of 21°C, and with a normal 12 h light-dark cycle (lights on: 08.00 h. to 20.00 h). Food (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and tap water were available *ad libitum*. One week before the experiment, cortical EEG electrodes were implanted under anaesthesia with 0.8 ml kg^{-1} of Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and 0.25 ml kg^{-1} Nembutal® (Sanofi, Maassluis, The Netherlands) as described before (Mandema & Danhof, 1990). During fixation in a stereotactic device four holes were drilled into the skull, without affecting the dura. The locations were 11 mm anterior and −2.5 mm lateral (F_1), 5 mm anterior and 3.5 mm lateral (C_r), 3 mm anterior and −3.5 mm lateral (C_1) to lambda. A little hole was made at 2.5 mm behind lambda to place the reference electrode. The electrodes consisted of stainless steel screws which were connected to a miniature connector (MS 333/3-A, Plastics One, Roanoake, Virginia, U.S.A.). The connector was fixed to the skull with dental acrylic cement. One day before the pharmacokinetic-pharmacodynamic experiment, cannulae were implanted in the right femoral artery (4.5 cm polythene tubing i.d. 0.28 mm heat-sealed to 18 cm polythene tubing i.d. 0.58 mm) and right jugular vein (12 cm polyvinylchloride tubing i.d. 0.5 mm), for the collection of arterial blood samples and drug administration respectively. The protocol of this study was approved by the Committee on Animal Experimentation of Leiden University.

Animal studies

The influence of the rate of rise in blood-concentration on the pharmacodynamics of midazolam was investigated in male Wistar rats, which were randomly allocated to six groups, four to eight animals per group, that were expected to receive a linear increase in blood concentration from 0–150 ng ml^{-1} in 1, 1.5, 2, 3, 4 and 6 h, respectively, using a computer controlled infusion pump (Harvard Apparatus Pump 22, South Natick, MA, U.S.A.). In addition in one group ($n=6$) the concentration was instantaneously brought to 150 ng ml^{-1} and maintained at this concentration for 4 h (clamp). A final treatment group ($n=9$) received a saline infusion for 4 h. The predicted concentration versus time profiles in the different treatment groups are summarized in Figure 1. Immediately following the infusion, an intravenous bolus dose of midazolam (10 mg kg^{-1} in 5 min) was administered. Midazolam was dissolved in 0.9% saline with the aid of an equimolar quantity of hydrochloric acid. In order to describe the concentration-time profile of midazolam arterial blood samples of 100 μl were withdrawn before intravenous bolus administration and at 2.5, 5, 7.5, 10, 15, 22.5 (50 μl), 30, 55, 90, 115 (100 μl), 150 and 175 (200 μl) min after intravenous bolus administration. The blood samples were immediately hemolyzed in 1 ml water and stored at −30°C until analysis.

The plasma-to-blood ratio (P/B) and the extent of plasma protein binding (% free fraction, fu) of midazolam were determined in each individual rat. Twenty-four hours after drug administration, a 3 ml blood sample was collected by aortic puncture. This blood sample was spiked with midazolam to a final concentration of 0.4 μg ml^{-1} of midazolam. The sample was centrifuged at 3000 r.p.m. (10 min, 20°C) to obtain plasma.

Using the YMT ultrafiltration membrane (Centrifree, Amicon, Beverly, MA, U.S.A.) a volume of 1 ml supernatant was ultrafiltrated within 40 min at 1090 *g* at 37°C to separate free midazolam from protein bound midazolam. Drug concentrations in whole blood, plasma and ultrafiltrate were determined by HPLC analysis as described below. Since protein binding of midazolam has been reported to be independent of concentration over a wide concentration range (0.01–10 mg l^{-1}; Moschitto & Greenblatt, 1983), plasma protein binding was determined at only one concentration.

Computer controlled infusion

In the experiment in which the blood concentration was clamped to a constant value, a computer controlled infusion pump (CCIP) was used (Harvard Apparatus Pump 22, Harvard Apparatus, South Natick, MA, U.S.A.). The pump was connected to a 80286 personal computer running Ms-Dos using a serial interface. The CCIP was designed to obtain and maintain a constant plasma drug concentration (clamp). For that purpose a modification of the algorithm originally proposed by Schwilden (1981) was used. First, the target concentration (C_T) is instantaneously achieved by administering a bolus dose equal to $C_T.V_1$, in which V_1 is the volume of the central compartment (0.39 l kg^{-1}). Simultaneously with

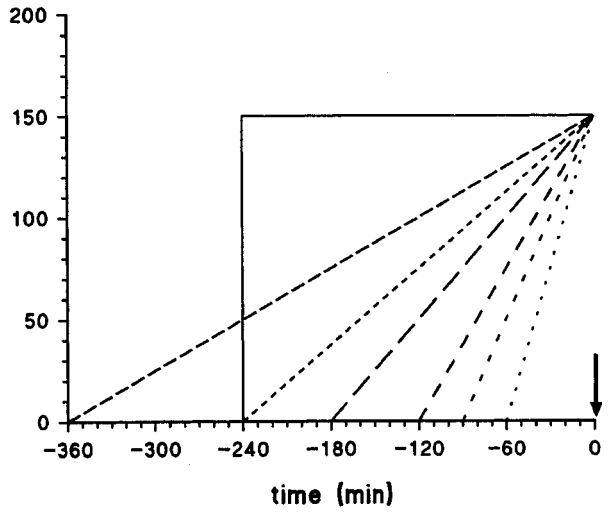

Figure 1 Predicted concentration *versus* time profiles during the first 4–6 h of the experiment. The arrow marks the start of the midazolam bolus infusion at the end of the pretreatment (10 mg kg^{-1}, 5 min). The linear increase in rate of rise in blood concentrations during the first 6, 4, 3, 2, 1.5, 1 h, are represented by the dashed lines, whereas the closed line represents the 4 h treatment at the EC_{50} concentration of 150 ng ml^{-1}.

the bolus infusion, a maintenance infusion is started. The rate of the maintenance infusion is calculated on the basis of the following equation:

$$R(t) = C_T V_1 (k_{10} + k_{12^e}^{-k_{21}t})$$

In this equation t is the time from the initial bolus dose, V_1 is the volume of the central compartment, k_{10} is the elimination rate constant (0.2710 min^{-1}) and k_{12} (0.3194 min^{-1}) and k_{21} (0.0470 min^{-1}) are the microconstants characterizing the distribution in the two-compartment pharmacokinetic model of midazolam. The values of the various pharmacokinetic parameters were taken from a previous study in the same strain of rats (Hoogerkamp *et al*., 1996).

The computer program TCIHARV (F. Engbers, Department of Anaesthesia, Leiden University Medical Center, Leiden, The Netherlands), was used to obtain a linear increase in the concentration of midazolam. In this instance the infusion rate was calculated according to the following equation:

$$R(t) = V_1 (S - S.t(k_{10} + k_{21^e}^{-k_{21}t})$$

in which S is a constant which is dependent on the concentration to be reached and the time at which this concentration should be reached (S = dC/dt), the other parameters are the same as described above. The software adjusted the infusion rate every 10 s to maintain the desired rate of increase in blood concentration. All infusion rates were recorded on the hard disk to provide a precise record of the drug infusion.

EEG monitoring

The EEG signal was recorded between F_1 and C_1 during the time course of the experiment using a DAM 50 differential amplifier (WPI, Sarato, U.S.A.), with the low pass filter set at 0.1 Hz and the high pass filter at 100 Hz. Subsequently the EEG signals were passed through a low pass filter set at 30 Hz, 70 db/oct (Department of Physiology, Leiden University, Leiden, The Netherlands). The electric signals were passed through a BNC/16 interface (Viewdac, Keithly, U.S.A.) into a IBM 40486 computer. The viewdac EEG analysis system (Viewdac, Keithly, U.S.A.) was used for data acquisition. The signals were subjected to on-line Fast Fourier Transform analysis for quantification. All experiments started between 09.00 h and 09.30 h in order to exclude diurnal variation in baseline pharmacodynamics. Recordings started 30 min before drug administration.

Analysis of drug concentration

The concentrations of midazolam in blood, plasma and ultrafiltrate were determined according to a HPLC method with u.v. detection, described by Mandema *et al*. (1991b) with some modifications. Calibration standards were prepared by adding 100 μl of midazolam solutions to 100 μl blood hemolyzed in 0.5 ml water to produce a blood concentration range of 25–5000 ng ml^{-1}. The standard midazolam solutions were prepared in methanol and were stable over a period of 3 months when stored at −4°C.

Diazepam (DZP) (80 ng in 50 μl methanol) was added to the sample as internal standard. The samples were further diluted with 750 μl 0.1 N NaOH. The samples were extracted after mixing with 5 ml petroleumether 40–60/dichloromethane (55:45 v v^{-1}) for 60 s on a vortex mixer. After centrifugation for 10 min at 2000 × *g*, the aqueous layer was frozen (20 min, −30°C). The organic layer was separated and evaporated to dryness under reduced pressure at 40°C. The residue was reconstituted in 150 μl of mobile phase and 50 μl were injected into the HPLC system. The liquid chromatographic system consisted of a Waters 510 HPLC pump, a Waters 717^{plus} automatic sample injector (Millipore Waters, Milford, MA, U.S.A.), a Spectroflow 757 variable-wavelength u.v. detector (Applied Biosystems, Ramsey, NJ, U.S.A.). Chromatography was performed at room temperature using an Alltima C_{18} 5 μm column (100 × 4.6 mm i.d. Alltech Associates, Deerfield, IL, U.S.A.) equipped with a guard column (20 × 2 mm i.d., Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with C_{18} (particle size 20–40 μm, Chrompack Nederland BV). Midazolam and the internal standard (DZP) were detected by u.v. absorption (λ = 222 nm). Data processing was performed on a Chrompack C-R3A reporting integrator (Shimadzu, Kyoto, Japan). The mobile phase consisted of a 46/54 (v v^{-1}) mixture of acetonitrile and 10 mM phosphate buffer (pH 7.5) delivered at a flowrate of 0.8 ml min^{-1}. Retention times were 4.5 and 5.4 min for DZP and midazolam, respectively. Peak height ratios of midazolam/diazepam were calculated and calibration curves were constructed by weighted linear regression (weight factor $(\text{peak-height ratio})^{-2}$). The calibration curves were linear ($r > 0.998$) and the intra-assay variations were 8 and 3% for blood concentrations of 50 and 100 ng ml^{-1}, respectively ($n = 6$), whereas the inter-assay variations were 4 and 2% for the previously mentioned concentrations. The limit of detection was 10 ng ml^{-1} for a sample of 50 μl blood as determined at a signal-to-noise ratio of 3.

Chemicals

Midazolam was a gift from Dr P. Weber (F. Hoffmann-La Roche AG, Basel, Schweiz) and Dr K. Dingelhoff (Roche Nederland B.V., Mijdrecht, The Netherlands). Dichloromethane and petroleumether 40–60 were purchased from Baker Chemicals (Deventer, The Netherlands) and distilled prior to use. Acetonitrile (HPLC grade) was obtained from Labscan (LabScan Ltd., Dublin, Ireland). Water was supplied by a Milli-Q system (Millipore SA, Molsheim, France). All other chemicals used were of analytical grade (Baker, Deventer, The Netherlands).

Data analysis

The rate of increase in blood concentration, as well as the pharmacokinetics and pharmacodynamics were quantified for each individual rat. The rate of increase in blood concentration was determined by linear regression of the blood concentration data during the first 1–6 h of the infusion. The blood concentration-time profiles after the bolus dose of midazolam were described by a poly-exponential equation for intravenous infusion:

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \cdot T}(1 - e^{-\lambda_i \cdot t}) \qquad (t < T) \qquad (1A)$$

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \cdot T}(e^{-\lambda_i \cdot (t-T)} - e^{-\lambda_i \cdot t}) \qquad (t \geq T) \qquad (1B)$$

where C(t) is the blood concentration of midazolam at time t, T the duration of the infusion and C_i and λ_i the coefficients and exponents of the equation respectively. Different models were investigated and tested according to the Akaike Information Criterion (Akaike, 1974; Yamaoka *et al*., 1978). In the

modelling procedure a weight factor of y^{-2} was used. Various pharmacokinetic parameters, such as clearance (Cl), volume of distribution at steady-state (Vdss) and elimination half-life ($t_{½}$), were calculated by standard methods from the coefficients and the exponents of the fitted functions using the combined data from the pretreatment period and the bolus infusion (Gibaldi & Perrier, 1982).

After the administration of the intravenous bolus dose, the sigmoidal E_{max} model was used to describe the relationship between midazolam concentration and EEG effect (Holford & Sheiner, 1982):

$$E_c = E_0 + \frac{E_{max} \cdot C^n}{EC^n{}_{50} + C^n} \qquad (2)$$

in which E_C is the EEG effect at midazolam blood concentration C, E_0 the baseline effect, E_{max} the maximum effect, EC_{50} the midazolam blood concentration at 50% of the maximum effect (E_{max}) and n the Hill factor, reflecting the sigmoidicity of the curve. In the modelling procedure E_0 was constrained to the average level of 20 min of baseline EEG recording before drug administration. EC_{50} values based on free drug concentration ($EC_{50,u}$) were obtained after correction for binding of midazolam to blood cells and plasma proteins.

The pharmacokinetic and pharmacodynamic data were analysed using the nonlinear least-square regression program Siphar version 3.0 (Simed Sa, Creteil, France). Pharmacokinetic and pharmacodynamic parameter estimates for the different administration rates were statistically evaluated using a one-way analysis of variance (ANOVA). In case of non-homogeneity of variances, as determined by Bartlett's test, the nonparametric least square differences test was used. A significance level of 5% was selected. Unless otherwise indicated, all data are reported as the mean ± s.e.mean.

Results

A blood concentration-time profile of midazolam for an individual rat during and after a 4 h exponential infusion is shown in Figure 2A. During the pretreatment infusion of 4 h a linear increase in concentration was seen. After the administration of the bolus dose, the pharmacokinetics could be described by a two compartment model.

The different concentration-time profiles during the first hours for three of the six groups are shown in Figure 3. The exponential infusions indeed produced a linear increase in blood concentration. In the various treatment groups receiving exponential infusions the mean values of the blood concentration at the end of the infusion periods were identical, 84 ± 6 ng ml^{-1} (mean ± s.d., n = 37). In the clamped group a stable blood concentration of 83 ± 6 ng ml^{-1} (mean ± s.d., n = 6) was reached within 10 min and maintained for 4 h. The observed rates of increase of blood concentration as calculated from the slopes of the curves of the individual animals for the different groups are listed in Table 1.

For all rats in the different groups a bi-exponential function satisfactorily described the time course of blood concentration after the administration of the bolus dose. The pharmacokinetic parameter estimates for the different infusion rate groups are summarized in Table 2. No significant differences between the groups of the different pretreatment protocols were observed. The average values of the total blood clearance, volume of distribution at steady-state and elimination half-life were 100 ± 3 ml min^{-1} kg^{-1}, 2.4 ± 0.1 l kg^{-1} and 28 ± 1 min respectively (n = 52).

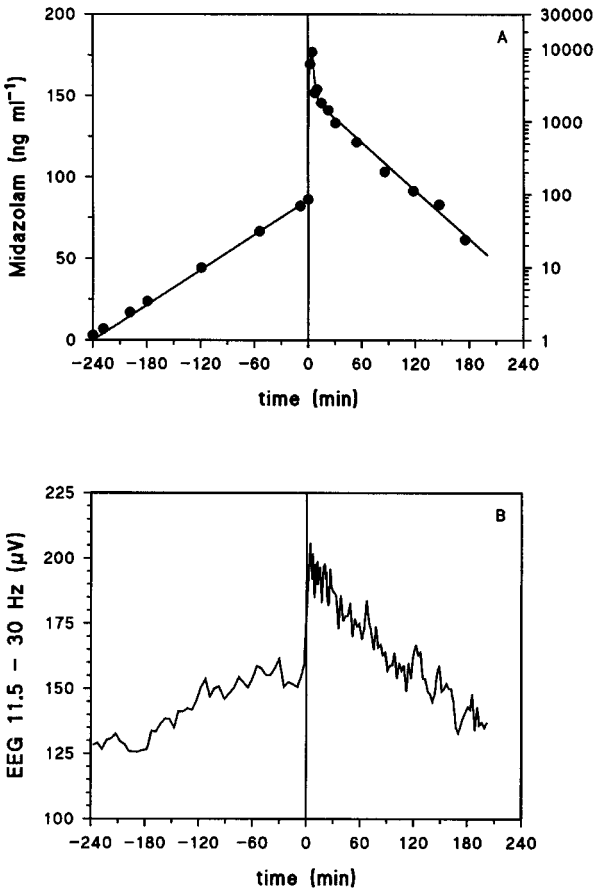

Figure 2 Blood concentration (A) and EEG effect (B) time profiles for a typical rat which was expected to receive a rate of rise of increase of midazolam concentration from 0–150 ng ml^{-1} in 4 h and a intravenous bolus dose of 10 mg kg^{-1} midazolam in 5 min. A bi-exponentional equation was fitted to the midazolam concentration data after the administration of the bolus dose. (N.B. The first 4 h of the infusion are plotted on a linear scale, whereas the concentrations after administering the bolus dose are plotted on a semi-logarithmic scale).

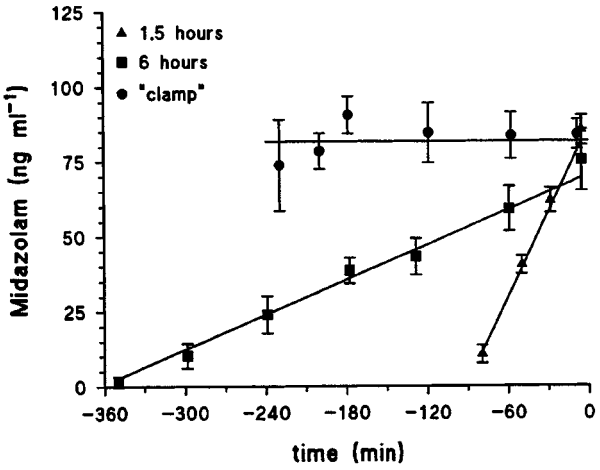

Figure 3 Observed midazolam blood concentration *versus* time profiles upon exponential infusion upon different administration rates. The straight lines represent the fits on basis of linear regression analysis.

The overall values of the plasma-to-blood ratio (P/B) and the free fraction were 1.23 ± 0.03 and 6.1 ± 0.5%, (n = 52) respectively. For the placebo group and the group with a rate

Table 1 Observed rates of rise in midazolam concentration, as calculated by linear regression analysis from the slope of the individual curves

Duration of the exponential infusion	*Predicted rate of change* (ng ml^{-1} min^{-1})	*Measured rate of change* (ng ml^{-1} min^{-1})	*n*
1 h	2.1	1.25±0.12	8
1.5 h	1.5	1.00±0.19	4
2 h	1.0	0.87±0.08	7
3 h	0.7	0.46±0.04	7
4 h	0.5	0.34±0.02	7
6 h	0.3	0.20±0.07	4

Table 2 Effect of the different rates of rise in midazolam concentration on the pharmacokinetic parameters of midazolam

Rate of rise in midazolam concentration (ng ml^{-1} min^{-1})	*Cl* (ml min^{-1} kg^{-1})	*Vdss* (l kg^{-1})	$t_{½}$ (min)
Placebo	111±8	2.5±0.14	26±1.2
Clamp	112±5	2.5±0.11	26±1.5
1.25	91±7	2.3±0.13	28±1.3
1.00	99±15	2.0±0.18	31±1.8
0.87	89±9	2.3±0.13	29±3.5
0.46	96±4	2.4±0.15	27±1.6
0.34	105±5	2.5±0.16	27±2.0
0.20	94±4	2.5±0.18	33±1.8

of rise in midazolam concentration of 0.34 ng ml^{-1}min^{-1}, the P/B ratios were 1.4±0.04 and 1.5±0.10, (n=14) respectively. These values were significantly different from the other groups (P<0.01). The change in the EEG effect (amplitude in the 11.5–30 Hz frequency band (β) band of the EEG power spectrum) and the drug concentrations in blood as function of time in an individual rat are shown in Figure 2B. During the first 4 h, the amplitude in the β activity of the EEG increased slowly. Immediately after the administration of the bolus dose of midazolam (10 mg kg^{-1}, t=0), a rapid and pronounced increase in the amplitude of the 11.5–30 Hz frequency band of the EEG was observed. The effect reached a maximum and maintained that level for some time after termination of the infusion. The effect then gradually returned to baseline values. No time delay (hysteresis or proteresis) between blood concentration and EEG effect was observed in any of the treatment groups, and the two were correlated directly to each other by the sigmoidal E_{max} model. In Figure 4 the concentration–EEG effect relationship after two different pretreatments is shown. The averaged pharmacodynamic parameter estimates obtained from the individual rats are represented in Table 3. Significant differences were observed for the $EC_{50,u}$ values of different pretreatment groups. The grouped data suggested that a critical range of rates of rise in midazolam concentrations (0.87 and 0.46 ng ml^{-1}min^{-1}) exists at which there is an increase in the $EC_{50,u}$ values, whereas slower and faster rates of rise in midazolam concentration do not influence the observed value of the $EC_{50,u}$. In order to examine this in more detail, the individual values of the $EC_{50,u}$ were plotted against the corresponding values of the rate of rise in midazolam concentrations (dC/dt). The result is shown in Figure 5. A biphasic (bell-shaped) relationship between $EC_{50,u}$ and the rate of rise in midazolam

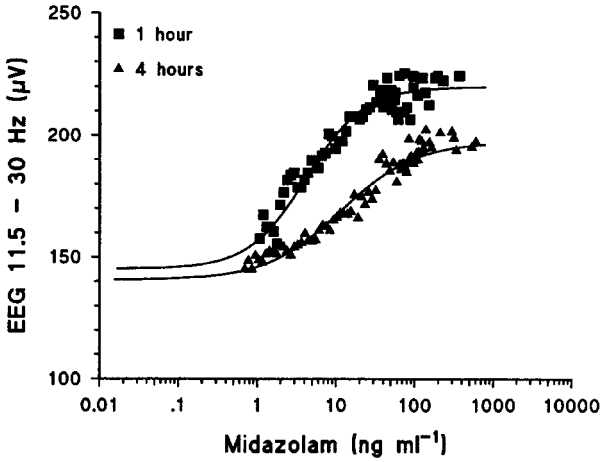

Figure 4 Midazolam concentration–EEG effect relationship for two typical rats which received different rates of rise of increase in midazolam concentration from 0–150 ng ml^{-1} followed by a bolus dose of 10 mg kg^{-1} midazolam in 5 min. The solid line represents the best fit to the actual data points according to the sigmoidal E_{max} model.

Table 3 Effect of the different rates of rise in midazolam concentration on the pharmacodynamic parameters of midazolam. (mean±s.e.mean)

Rate of rise of midazolam concentration (ng ml^{-1} min^{-1})	E_0 (μV)	E_{max} (μV)	$EC_{50,u}$ (ng.ml^{-1})	*Hill slope*
Placebo	168±6	82±5	6.4±0.8	1.4±0.1
Clamp	124±6*	91±10	7.7±0.2	1.2±0.2
1.25	156±6	83±6	7.4±1.0*	1.3±0.1
1.00	174±19	100±11	10±1.8	1.6±0.3
0.87	145±8*	76±8	19±4.4*	1.3±0.2
0.46	137±3*	59±3*	21±5.0*	1.2±0.2
0.34	116±6*	57±5*	12±2.5	1.0±0.2
0.20	171±5	65±8	10±3.2	1.5±0.4

*P<0.05 *versus* placebo.

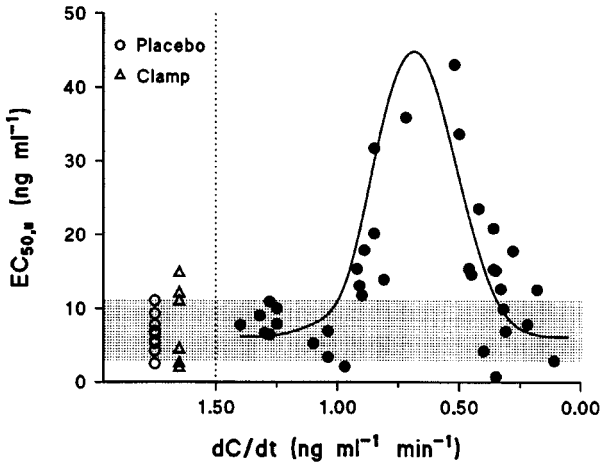

Figure 5 Relationship between the individual $EC_{50,u}$ values and the rate of increase in midazolam concentration. The shaded area in the figure presents the 95% confidence interval of the $EC_{50,u}$ values in the placebo group. The curve was not fitted to the data but drawn to show the bell-shape form (n=37).

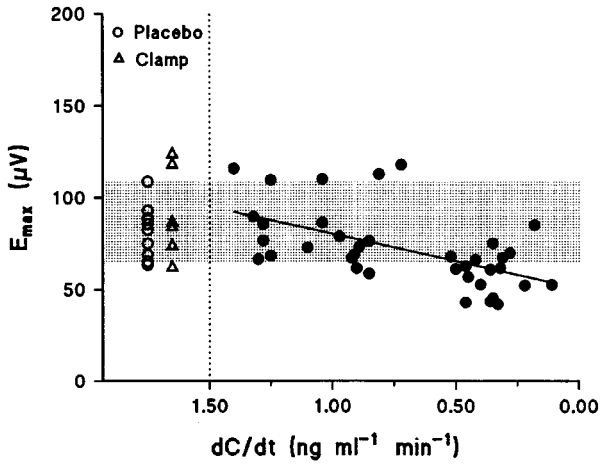

Figure 6 Plot of the intrinsic efficacy (E_{max}) *versus* the rate of increase in midazolam concentration for each individual animal. The shaded area in the figure presents the 95% confidence interval of the E_{max} values in the placebo group. The solid line represents the regression line: $Y = 50.6 + 29.8$ (dC/dt); ($r^2 = 0.327$, $n = 37$).

concentration was observed with values that largely exceed the 95% confidence interval observed in the placebo group. The individual with the highest value of the $EC_{50,u}$ of 43 ng ml^{-1} was observed at the rate of rise of the midazolam blood concentration of 0.48 ng ml^{-1}min^{-1}.

The values of the E_{max} were similar in the different treatment groups although there appeared to be a tendency towards a decrease at the lower infusion rates. In the clamped group, in which the blood concentration of midazolam was maintained at 83 ng ml^{-1} during 4 h, the value of E_{max} was identical to the value in the placebo group. Investigation of the individual E_{max} values *versus* the rate of rise of midazolam concentrations revealed a weak tendency to lower E_{max} values with decreasing rate of midazolam concentration ($r^2 = 0.327$; Figure 6), although most of the values are still within the 95% confidence interval observed in the placebo group. No relevant differences in E_0 and Hill factor were observed for any of the different pretreatments.

Discussion

Acute tolerance towards several actions of benzodiazepines in man and animals has been reported (Breimer *et al*., 1992; Ellinwood *et al*., 1983; Smith & Kroboth, 1987; Kroboth *et al*., 1988). It has been suggested that the rate of drug administration may be an important determinant of the clinal effects of benzodiazepines and, in particular the development of acute tolerance (Ellinwood *et al*., 1985; Greenblatt *et al*., 1977; Grundström *et al*., 1978; Kroboth *et al*., 1988). So far however, no quantitative information is available on the relationship between the rate of change in drug concentration on one hand and the values of quantitative pharmacodynamic parameters on the other. The objective of the present investigation was to characterize quantitatively, the influence of the rate of change of blood concentration on the pharmacodynamics of benzodiazepines, using computer controlled infusion regimens, which result in a constant rise of the blood concentration over time. The results of this investigation show not only that the pharmacodynamics of midazolam are highly dependent on the rate of rise in blood concentrations, but furthermore that the relationship between the rate of change of blood concentration and the values of the different pharmacodynamic parameters is rather complex. A biphasic relationship between the $EC_{50,u}$ and the rate of change in blood concentrations was observed.

In several studies it has been suggested that the rate of drug administration may be an important determinant of the pharmacodynamics of benzodiazepines (Greenblatt *et al*., 1977; Grundström *et al*., 1978; Ellinwood *et al*., 1985; Kroboth *et al*., 1988). A limitation of these studies is however, that the investigators have not been able to accurately characterize and control the rate of change of drug concentrations. In most studies zero-order infusion rates were used for the administration of the benzodiazepines. By using a zero-order infusion however, the rate of rise of blood concentration continuously varies with time, as the concentration approaches steady-state. This makes it very difficult, if not impossible to draw conclusions about the relationship between the rate of change of drug concentrations and the pharmacodynamics of benzodiazepines. The recent advances in characterizing the pharmacokinetics of benzodiazepines in rats (Mandema *et al*., 1991a,b; 1992a,d; Hoogerkamp *et al*., 1996) as well as development of novel computer controlled infusion techniques, made it possible to carefully control the blood concentration-time profiles (Gustafsson *et al*., 1992). The present investigation shows that by applying this new methodology, indeed a linear increase in blood concentration of midazolam can be obtained. The observed rates of rise in blood concentration were 1.25, 1.00, 0.87, 0.46, 0.34, 0.20 ng ml^{-1}min^{-1}, respectively. These rates of rise in blood concentrations were lower than predicted on the basis of the pharmacokinetic models (Table 1). This can be explained by the difference between the observed values of pharmacokinetic parameters (Table 2) and the values that were used in the programming of the infusion pump. For the programming of the infusion pump the values of Hoogerkamp *et al.* (1996) for clearance and volume of distribution were used of 62 ± 4 ml min^{-1}kg^{-1} and 2.7 ± 0.1 l kg^{-1}. The observed values were significantly different. Between different studies, even from the same laboratory, wide differences in the pharmacokinetic parameters of midazolam have been observed. Following intravenous infusion of a midazolam bolus dose, values of the clearance varying between (mean $\pm$ s.e.mean) of 60 ± 2 and 99 ± 10 ml min^{-1}kg have been reported, whereas the values of the volume of distribution at steady-state vary between 1.5 ± 0.1 and 2.7 ± 0.2 l kg^{-1} and those of the half-life between 24 ± 1 and 31 ± 3 min (Mandema *et al*., 1991a,b; 1992a,d; Hoogerkamp *et al*., 1996). The values of the pharmacokinetic parameters observed in the present study, although significantly different from those reported by Hoogerkamp *et al.* (1996) are still within this range. At present there is no clear explanation why the values of the pharmacokinetic parameters differ between different studies.

In the present study amplitude in the 11.5–30 Hz (β) frequency band of the EEG effect was used as a pharmacodynamic measure. This parameter fulfils many of the criteria of an ideal pharmacodynamic measure in that it provides a continuous, objective, reproducible and sensitive measure of the pharmacological response intensity (Dingemanse *et al*., 1988). Furthermore the pharmacological relevance of this measure has recently been convincingly demonstrated. For a wide range of different benzodiazepines a close correlation between *in vivo* potency ($EC_{50,u}$) and the affinity to the GABA-benzodiazepine receptor complex *in vitro* was observed (Mandema *et al*., 1991a). Furthermore, between benzodiazepines receptor full agonists, partial agonists and inverse agonists there were important differences in E_{max} (Mandema *et al*., 1992a). These findings justify the conclusion that

amplitude in the 11.5–30 Hz frequency band of the EEG is indeed a relevant measure of effect of benzodiazepines *in vivo*, reflecting both differences in affinity to and intrinsic efficacy at the GABA-benzodiazepine receptor complex (Mandema & Danhof, 1992).

The concentration EEG-effect relationship of midazolam was studied after different pretreatments. No differences in intrinsic efficacy (E_{max}) nor *in vivo* potency ($EC_{50,u}$) were found between the clamp and placebo group. This indicates that acute tolerance did not develop towards the EEG effects of midazolam in rats, even after prolonged exposure to concentrations of 83 ± 5 ng ml^{-1}, which is around the EC_{50}. A tendency towards a decrease in maximum effect (E_{max}) was observed with a slow rate of rise in blood concentrations although most of the observed E_{max} values are still within the 95% confidence interval of the placebo group. For the $EC_{50,u}$ however a profound effect of the rate of rise of the blood concentration was observed. The relationship between the rate of rise of the blood concentration and the $EC_{50,u}$ was quite complex. When the blood concentration of midazolam is rapidly increased to a value around the EC_{50} (83 ± 6 ng ml^{-1}) and maintained at that value for 4 h ('clamp'), values of the $EC_{50,u}$ are observed which are still within the 95% confidence interval of the placebo group. This observation is in agreement with the results of the studies of Mandema *et al.* (1992b,c) where upon rapid increase of the blood concentration to a steady-state level around the EC_{50} also no change in the pharmacodynamic parameter estimates was observed. At different rates of increase of the blood concentration however, values of the $EC_{50,u}$ are observed that largely exceed the 95% confidence interval in the placebo group. Interestingly the relationship between the rate of rise of the blood concentration and the value of the $EC_{50,u}$ is biphasic. A certain critical range of the rate of rise of the blood concentration is observed at which the $EC_{50,u}$ is particularly sensitive to change. This change in the $EC_{50,u}$ is purely pharmacodynamic in nature, as in previous investigations it has been convincingly demonstrated that potentially complicating pharmacokinetic factors such as distribution to the site of action and formation of interactive metabolites do not interfere with the assessment of the pharmacodynamics of midazolam in rats *in vivo* when using an integrated pharmacokinetic/pharmacodynamic approach (Mandema *et al.*, 1991a). The observed biphasic relationship between EC_{50} and rate of change in blood concentrations can explain the observed co-variation between the increase in EC_{50} for the sedative effects of midazolam and the decrease in absorption rate constant after different s.c. doses of midazolam in the rat (Lau *et al.*, 1998), on the assumption that the absorption rate constant reflects the rate of change in blood concentrations. Furthermore, the findings of the present study indicate that *in vivo* a homeostatic control mechanism is operative which may modify the sensitivity to midazolam and of which the activation is largely influenced by the rate of presentation of the drug. Apparently at very rapid rates of presentation of the drug, this system is unable to adapt to the perceived drug effect ('freezing' of the system) whereas at very low rates drug presentation (and therefore a very low rate of change of the effect) the system is not activated. At intermediate rates of administration however the system is activated and counteracts the effect. Thus acute functional tolerance development to midazolam is clearly dependent on the rate of presentation of the drug. Therefore, it can be hypothesized that the effect of midazolam may vary with the route of administration, since different routes of drug administration may result in different absorption rate constants and thereby in different rates of change in blood concentrations.

Interestingly in man, acute tolerance development has been reported to the EEG effect of midazolam (Breimer *et al.*, 1992) and to the psychomotor effects of alprazolam (Kroboth *et al.*, 1988) and triazolam (Kroboth *et al.*, 1993) . In these studies the subjects received a 'loading' dose of 2–10 min followed by a constant-rate infusion to maintain a constant plasma concentration. Thereby however a relative overshoot in the steady-state concentration occurred during the administration of the loading infusion. Furthermore, the loading dose was administered with a zero-order infusion and therefore a changing rate of rise in plasma concentrations was applied to achieve steady-state plasma concentrations. One possible explanation for the observed difference in acute tolerance development may therefore be the difference in rate of rise in plasma concentrations. However, differences in metabolic pathways between the two species, rat and man, and the interference of different (inter)active metabolites must be considered too. In contrast to the rat, in man metabolites of midazolam were found to contribute to the pharmacological effects of the drug (Crevoisier *et al.*, 1983; Mandema *et al.*, 1992d). It has been suggested that the apparent acute tolerance to the actions of diazepam in man is due to redistribution of the compound from the central nervous system to the peripheral tissues (Greenblatt & Shader, 1986). Acute tolerance to the hypnotic effect of diazepam in mice may not be attributed to changes in pharmacokinetic factors (Yoong *et al.*, 1986).

Numerous changes at the GABA-benzodiazepine receptor complex in response to chronic occupation of the benzodiazepines binding site have been reported. Some of these changes include internalization of the receptor, a subsensitivity to GABA, an uncoupling of sites at the GABA receptor complex (Wilson & Gallager, 1988; Roca *et al.*, 1989; Tehrani & Barnes, 1991). Other mechanisms that might be involved are alterations in levels of GABA receptor subunit mRNA and receptor phosphorylation (Kang & Miller, 1991; Gyenes *et al.*, 1994; Wu *et al.*, 1994). Besides upon long-term treatment with benzodiazepines a reduction of electrophysiological response to GABA has been reported and it is known that exposure of neurons to muscimol, a GABA analogue, results in desensitization within seconds (Tietz *et al.*, 1989). Moreover, very recently it has been shown that short term exposure to flunitrazepam (1 h) *in vitro* leads to 20% decrease in α, and B2/3 $GABA_A$ receptor subunits (Johnston *et al.*, 1998). To what extent these processes contribute to the complex profile of the pharmacodynamics upon different rates of rise in drug concentration remains to be established.

In conclusion, the concept that the pharmacodynamics of benzodiazepines are dependent on the rate of rise in drug concentration is supported in this study. Although numerous investigators (Ellinwood *et al.*, 1985; Greenblatt *et al.*, 1977; Grundström *et al.*, 1978; Kroboth *et al.*, 1988) have suggested that the development of acute tolerance to the benzodiazepines depends on the rate of drug administration, this is the first study to demonstrate quantitatively that the rate of change in blood concentrations is a major determinant of the pharmacodynamics.

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References

AKAIKE, H. (1974). A new look at the statistical model identification. *IEEE Trans. Auto. Control,* **19,** 716–723.

ALVIS, J.M., REVES, J.G., SPAIN, J.A. & SHEPPARD, L.C. (1985). Computer-assisted continuous infusion of the intravenous analgesic fentanyl during general anaesthesia–an interactive system. *IEEE Trans. Biomed. Eng.,* **32,** 323–329.

AUSEMS, M.E., STANSKI, D.R. & HUG, C.C. (1985). An evaluation of the accuracy of pharmacokinetic data for the computer assisted infusion of alfentanyl. *Br. J. Anaesth.,* **57,** 1217–1225.

BREIMER, L.T.M., HENNIS, P.J., BURM, A.G.L., DANHOF, M., MANDEMA, J.W., VLETTER, A.A., BOVILL, J.G. & SPIERDIJK, J. (1992). EEG effect during pseudo 'steady state' infusion of midazolam in volunteers: development of acute tolerance? *Clin. Pharmacol. Ther.,* **51,** 188.

BÜHRER, M., MAITRE, P.O., EBLING, W.F. & STANSKI, D.R. (1987). Defining thiopental's steady-state plasma concentration–EEG effect relationship. *Anesthesiology,* **67,** A399.

COSTA, E. & GUIDOTTI, A. (1996). Benzodiazepines on trial: a research strategy for their rehabilitation. *Trends Pharmacol. Sci.,* **17,** 192–200.

CREVOISIER, C.H., ZIEGLER, W.H., ECKERT, M. & HEIZMANN, P. (1983). Relationship between plasma concentration and effect of midazolam after oral and intravenous administration. *Br. J. Clin. Pharmacol.,* **16,** 51S–61S.

DINGEMANSE, J., DANHOF, M. & BREIMER, D.D. (1988). Pharmacokinetic pharmacodynamic modelling of CNS effects. An overview. *Pharmacol. Ther.,* **38,** 1–52.

ELLINWOOD, JR, E.H., HEATHERLY, D.G., NKAIDO, A.M., BJORNSSON, T.D. & KILTS, C. (1985). Comparative pharmacokinetics and pharmacodynamics of lorazepam, alprazolam and diazepam. *Psychopharmacol.,* **86,** 392–399.

ELLINWOOD, JR, E.H., LINNOILA, M., EASLER, M.E. & MOLTER, D.W. (1983). Profile of acute tolerance to three sedative anxiolytics. *Psychopharmacol.,* **79,** 137–141.

GALLAGER, D.W., LAKOSKI, J.M., GONSALVES, S.F. & RAUCH, S.L. (1984). Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. *Nature (Lond.),* **308,** 74–77.

GALLAGER, D.W., MALCOLM, A.B., ANDERSON, S.A. & GONSALVES, S.F. (1985). Continuous release of diazepam: electrophysiological, biochemical and behavioral consequences. *Brain Res.,* **342,** 26–36.

GIBALDI, M. & PERRIER, D. (1982). Pharmacokinetics 2nd ed., Marcel Dekker Inc. New York.

GREENBLATT, D.J. & SHADER, R.I. (1986). Long-term administration of benzodiazepines: pharmacokinetic versus pharmacodynamic tolerance. *Psychopharmacol. Bulletin,* **22,** 410–423.

GREENBLATT, D.J., SHADER, R.I., HARMATZ, J.S., FRANKE, K. & KOCH-WESER, J. (1977). Absorption rate, blood concentrations, and early response to oral chlordiazepoxide. *Am. J. Psychiatry,* **134,** 559–562.

GRUNDSTRÖM, R., HOLMBERG, G. & HANSEN, T. (1978). Degree of sedation obtained with various doses of diazepam and nitrazepam. *Acta Pharmacol. Toxicol.,* **43,** 13–18.

GUSTAFSSON, L.L., EBLING, W.F., OSAKI, E., HARAPAT, S., STANSKI, D.R. & SHAFER, S.L. (1992). Plasma concentration clamping in the rat using a computer-controlled infusion pump. *Pharm. Res.,* **9,** 800–807.

GYENES, M., WANG, Q., GIBBS, T.T. & FARB, D.H. (1994). Phosphorylation factors control neurotransmitter and neuromodulator actions at the γ-aminobutyric acid type A receptor. *Mol. Pharmacol.,* **46,** 542–549.

HAIGH, J.R.M. (1988). Tolerance to the anticonvulsant effect of benzodiazepines. *Trends Pharmacol. Sci.,* **9,** 361–366.

HOLFORD, N.H.G. & SHEINER, L.B. (1982). Kinetics of pharmacologic response. *Pharmacol. Ther.,* **16,** 143–166.

HOOGERKAMP, A., ARENDS, R.H.G.P., BOMERS, A.M., MANDEMA, J.W., VOSKUYL, R.A. & DANHOF, M. (1996). Pharmacokinetic/pharmacodynamic relationship of benzodiazepines in the direct cortical stimulation model of anticonvulsant effect. *J. Pharmacol. Exp. Ther.,* **279,** 803–812.

JOHNSTON, J.D., PRICE, S.A. & BRISTOW, D.R. (1998). Flunitrazepam rapidly reduces $GABA_A$ receptor subunit expression *via* a protein C-dependent mechanism. *Br. J. Pharmacol.,* **124,** 1338–1340.

KANG, I. & MILLER, L.G. (1991). Decreased $GABA_A$ receptor subunit MRNA concentrations following chronic lorazepam administration. *Br. J. Pharmacol.,* **103,** 1285–1287.

KROBOTH, P.D., BERTZ, R.J. & SMITH, R.B. (1993). Acute tolerance to triazolam during continuous and step insions: Estimation of the effect offset rate constant. *J. Pharmacol. Exp. Ther.,* **264,** 1047–1055.

KROBOTH, P.D., SMITH, R.B. & ERB, R.J. (1988). Tolerance to alprazolam after intravenous bolus and continuous infusion: psychomotor and EEG effects. *Clin. Pharmacol. Ther.,* **43,** 270–277.

LAU, C.E., WANG, Y. & MA, F. (1998). Pharmacokinetic-pharmacodynamic modelling of the coexistence of stimulatory and sedative components for midazolam. *Eur. J. Pharmacol.,* **346,** 131–144.

MANDEMA, J.W. & DANHOF, M. (1990). Pharmacokinetic-pharmacodynamic modelling of the central nervous system effects of heptabarbital using aperiodic EEG analysis. *J. Pharmacokin. Biopharm.,* **5,** 459–481.

MANDEMA, J.W. & DANHOF, M. (1992). Electroencephalogram effect measures and relationships between pharmacokinetics and pharmacodynamics of centrally acting drugs. *Clin. Pharmacokin.,* **23,** 191–215.

MANDEMA, J.W., KUCK, M.T. & DANHOF, M. (1992a). Differences in intrinsic efficacy of benzodiazepines are reflected in their concentration–EEG effect relationship. *Br. J. Pharmacol.,* **105,** 164–170.

MANDEMA, J.W., KUCK, M.T. & DANHOF, M. (1992b). *In vivo* modelling of the pharmacodynamic interaction between benzodiazepines which differ in intrinsic efficacy. *J. Pharmacol. Exp. Ther.,* **261,** 56–61.

MANDEMA, J.W., SAMSON, L.N., DIOS-VIEITEZ, M.C., HOLLANDER-JANSEN, M. & DANHOF, M. (1991a). Pharmacokinetic-pharmacodynamic modelling of the electro-encephalogram effects of benzodiazepines. Correlation with receptor binding and anticonvulsant activity. *J. Pharmacol. Exp. Ther.,* **257,** 472–478.

MANDEMA, J.W., TUK, B., VAN STEVENINCK, A.L., BREIMER, D.D., COHEN, A.F. & DANHOF, M. (1992d). Pharmacokinetic-pharmacodynamic modelling of the CNS effects of midazolam and its main metabolite α-hydroxy-midazolam in healthy volunteers. *Clin. Pharmacol. Ther.* **51,** 715–728.

MANDEMA, J.W., TUKKER, E. & DANHOF, M. (1991b). Pharmacokinetic-pharmacodynamic modelling of the EEG effects of midazolam in individual rats: influence of rate and route of administration. *Br. J. Pharmacol.,* **102,** 663–668.

MANDEMA, J.W., TUKKER, E. & DANHOF, M. (1992c). *In vivo* characterization of the pharmacodynamic interaction of a benzodiazepine agonist and antagonist: midazolam and flumazenil. *J. Pharmacol. Exp. Ther.,* **260,** 36–44.

MARTIN, P. & AHUJA, A.A. (1988). Optimal pharmacokinetic delivery of infused drugs: Application to the treatment of cardiac arrhythmias. *J. Biomed. Eng.,* **10,** 360–384.

MILLER, L.G., GREENBLATT, D.J., BARNHILL, J.G. & SHADER, R.I. (1988). Chronic benzodiazepine administration: I Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric acid A receptor function. *J. Pharmacol. Exp. Ther.,* **246,** 170–176.

MOSCHITTO, L.J. & GREENBLATT, D.J. (1983). Concentration independent plasma protein binding of benzodiazepines. *J. Pharm. Pharmacol.,* **35,** 179–180.

POULOS, C.X. & CAPPELL, H. (1991). Homeostatic theory of drug tolerance: a general model of physiological adaptation. *Psychol. Rev.,* **98,** 390–408.

ROCA, D.J., ROZENBERG, I., FARRANT, M. & FARB, D.H. (1989). Chronic agonist exposure induces down-regulation and allosteric uncoupling of the γ-Aminobutyric acid/benzodiazepine receptor complex. *Mol. Pharmacol.,* **37,** 37–43.

SCHWILDEN, H. (1981). A general method for calculating the dosage scheme in linear pharmacokinetics. *Eur. J. Clin. Pharmacol.,* **20,** 379–386.

SMITH, R.B. & KROBOTH, P.D. (1987). Influence of dosing regimen on alprazolam and metabolite serum concentrations and tolerance to sedative and psychomotor effects. *Psychopharmacol.,* **93,** 105–112.

TACKLEY, R.M., LEWIS, G.T., PRYS-ROBERTS, C., BOADEN, R.W., DIXON, J. & HARVEY, J.T. (1989). Computer controlled infusion of propofol. *Br. J. Anaesth.,* **62,** 46–63.

TEHRANI, M.H.J. & BARNES, JR., E.M. (1991). Agonist dependent internalization of γ-aminobutyric $acid_A$/benzodiazepine receptors in chick cortical neurons. *J. Neurochem.,* **57,** 1307–1312.

TIETZ, E.I., CHIU, T.H. & ROSENBERG, H.C. (1989). Regional GABA/benzodiazepine receptor/chloride channel coupling after acute and chronic benzodiazepine treatment. *Eur. J. Pharmacol.,* **167,** 57–65.

WILSON, M.A. & GALLAGER, D.W. (1988). GABAergic subsensitivity of dorsal raphe neurons *in vitro* after chronic benzodiazepine treatment. *Brain Res.,* **473,** 198–202.

WU, Y., ROSENBERG, H.C., CHIU, T.H. & ZHAO, T.-J. (1994). Subunit- and brain region-specific reduction of $GABA_A$ receptor subunit MRNAS during chronic treatment of rats with diazepam. *J. Mol. Neurosc.,* **5,** 105–120.

YAMAOKA, K., NAKAGAWA, T. & UNO, T. (1978). Application of Akaike's Information Criterion (AIC) in the evaluation of linear pharmacokinetic equations. *J. Pharmacokin. Biopharm.,* **6,** 165–175.

YOONG, Y.L., LEE, H.S., GWEE, M.C.E. & WONG, P.H.-H. (1986). Acute tolerance to diazepam in mice: pharmacokinetic considerations. *Clin. Exp. Pharmacol. Physiol.,* **13,** 153–158.

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