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Effect of the anticonvulsant denzimol on the disposition of diazepam in the rat

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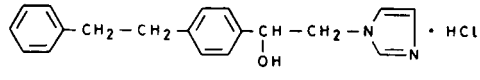
The influence of denzimol, a new imidazole derivative with anticonvulsant properties, on the disposition of diazepam (1 mg kg^{-1}) was investigated in the rat. Denzimol pretreatment significantly increased plasma and brain concentrations of the benzodiazepine, consistent with reduced total clearance. The results suggest that the increase of diazepam brain concentrations by denzimol may produce enhancement of diazepam activity in the rat.

Diazepam is a lipophilic compound which, like many benzodiazepine derivatives, is almost entirely eliminated from the body by biotransformation. Its elimination is initiated by P450-dependent phase I reactions involving, with species differences, N_1-demethylation and/or hydroxylation of the molecule (Schwartz et al 1965; Garattini et al 1973; Randall & Kappell 1973). These processes may be influenced by pathological and physiological variables which normally impair the drug elimination (Klotz et al 1975, 1977). Similarly, compounds which inhibit hepatic microsomal drug metabolism also impair the hepatic clearance of this benzodiazepine (Klotz et al 1979; Klotz & Reimann 1980, 1981; Abernethy et al 1982, 1985; Gugler & Jensen 1984).

In the course of pharmacological studies of denzimol *N*-[β-[4-(β-phenylethyl)phenyl]-β-hydroxyethyl]-imidazole hydrochloride (Mennini et al 1984), a newly developed anticonvulsant agent (Nardi et al 1981), it was noted that the drug potentiated some pharmacological effects of diazepam in rats. On the basis of biochemical studies, it was suggested that this potentiating effect involved some interaction with benzodiazepine receptors. Denzimol contains an imidazole moiety in its chemical structure (I), making it a potential



(I)

inhibitor of drug metabolism. Many imidazole derivatives inhibit hepatic mono-oxygenase function, and for some derivatives this effect may be mediated in part through direct binding to microsomal cytochrome P450 (Puurunen & Pelkonen 1979; Rendic et al 1979; Dickins & Bridges 1982; Hajek et al 1982; Kapetanovic & Kuppferberg 1984; Meredith et al 1985). Accordingly, the aim of this investigation was to assess whether the disposition of diazepam in the rat was altered by pretreatment with denzimol.

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Materials and methods

Animals and treatment. Male CD-COBS rats (Charles River, Italy), 270–300 g, were acclimatized to the research facility for one week before the study, and had free access to standard laboratory chow and water. Animal rooms had controlled temperature (22 °C) and light cycle (12/12 h). The kinetics of diazepam were studied after i.v. injection to six control rats and five given denzimol hydrochloride (5 mg kg^{-1} p.o., 15 min before diazepam injection) chronically cannulated via the right jugular vein. The length of polyethylene tubing was such that the tip of the cannula resided in the right atrium (confirmed by autopsy) and thus blood samples were taken from, and diazepam (1 mg kg^{-1}) was administered into the mixed venous blood supply. The cannula was flushed with 0·15 ml 0·9% NaCl (saline) and approximately 0·05 ml of blood was withdrawn and discarded. A minimum of eight 0·1 ml samples were taken over a period of approximately three diazepam half-lives (Klotz et al 1976; Caccia et al 1980; Igari et al 1982). The blood samples were collected in heparinized tubes, centrifuged and the plasma stored at −20 °C until analysis. Brains were rapidly removed at the end of the experiment (180 min) and stored at −20 °C. In separate experiments other groups of animals were killed 60 min after diazepam injection (1 mg kg^{-1}) and blood and brain samples processed as above.

The effect of denzimol on the plasma protein binding of diazepam was determined in-vitro by addition of denzimol (2·0 nmol ml^{-1}) and diazepam (0·2 and 2·0 nmol ml^{-1}) to pooled rat serum and dialysing the serum against phosphate buffer (pH 7·4) at 37 °C to equilibrium.

Chemical analysis. Diazepam and its active metabolites nordiazepam, *N*-methyloxazepam and oxazepam were determined by electron capture gas liquid chromatography. For extraction, 0·05 ml of plasma, 0·01 ml of a methanolic solution of *o*-chlorodiazepam (internal standard) and 1 ml benzene were mixed (15 min) with a Heidolph Reax shaker in Eppendorf microtubes. After centrifugation, approximately 0·9 ml of benzene were evaporated to dryness, redissolved in 0·1 ml of the solvent and 1 μl was injected into the chromatographic column.

Brains were homogenized (6 ml g^{-1}) in 0·1 M phosphate buffer pH 7·4, containing the internal standard (5 ng ml^{-1}), and then extracted with benzene. After

centrifugation a portion of the solvent layer (1 ml) was processed as above.

The gas chromatograph was a C. Erba Fractovap mod. 2150 equipped with a ^{63}Ni electron capture detector. The chromatographic column was a glass tube (1 m × 3 mm i.d.) packed with 80–100 mesh Chromosorb W(HP) with 3% OV-225 as the stationary phase (Pierce, Rockford, USA). The oven, injector port and detector temperatures were 245, 300 and 275 °C, respectively. The carrier gas was nitrogen at a flow-rate of 30 ml min^{-1}. Under these experimental conditions, approximate retention times were 2·0 min for oxazepam, 3·0 min for diazepam, 6·2 min for nordiazepam, 12·0 min for *N*-methyloxazepam and 4·2 min for *o*-chlorodiazepam. Fig. 1 shows a typical chromatogram of the extract from a spiked plasma sample. Calibration plots of peak area against concentration were linear for each compound over the range 0·05–1·0 nmol ml^{-1} or g^{-1}, with a coefficient of variation of 5–10%. Recoveries from plasma and tissues were in the range of 85–95%. The limit of detection was 0·05 nmol ml^{-1} or g^{-1} or better for diazepam and 0·05–0·01 nmol ml^{-1} or g^{-1} for its metabolites, extracting 0·05 ml of plasma and approximately 100 mg of brain tissue.

Data analysis. A biexponential equation of the form

$$C(t) = A\,e^{-\alpha t} + B\,e^{-\beta t} \qquad (1)$$

was fitted to the individual plasma diazepam concentration-time data, weighting each datum by the reciprocal of the square of the estimated concentration, using a non-linear regression analysis program (C. Peck, Uniformed Services University, Bethesda, USA) and run according to Sacchi Landriani et al (1983) on an HP-85 computer. All the kinetic parameters were calculated from equation (1) in the standard manner (Gibaldi & Perrier 1982). Statistical analysis was done by Student's *t*-test and probabilities (P) < 0·05 were considered statistically significant.

FIG. 1. Gas chromatogram of oxazepam (OX), diazepam (DZ), nordiazepam (DDZ), *N*-methyloxazepam (MOX) and the internal standard (ClDZ) from a spiked plasma sample.

Results

The mean plasma concentration-time curves of diazepam after i.v. injection (1 mg kg^{-1}) to control and denzimol-pretreated rats are shown in Fig. 2. Within 180 min the plasma concentrations of the benzodiazepine in controls fell from about 10 nmol ml^{-1} to near the limit of sensitivity of the analytical procedure; after pretreatment with denzimol (5 mg kg^{-1} p.o.) the plasma concentrations of diazepam were increased throughout the sampling period.

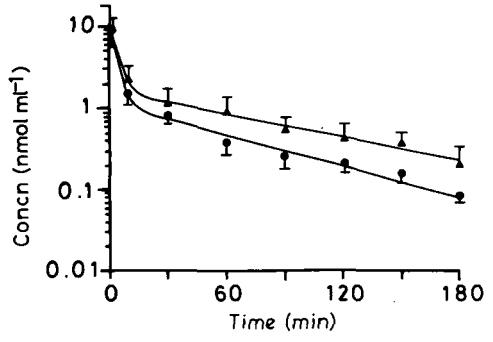

Fig. 2. Mean plasma concentration-time curves of diazepam after intravenous injection (1 mg kg^{-1}) to control and denzimol (5 mg kg^{-1} p.o.)-treated rats. Data are the mean ± s.d. for six control (●) and five denzimol (▲) -treated rats.

Some kinetic parameters derived from these figures are summarized in Table 1. The apparent volume of distribution (V_β) of diazepam ranged from about 2·4 litre kg^{-1} in controls to about 1·8 litre kg^{-1} in

Table 1. Disposition of diazepam in controls and denzimol-pretreated rats.

Parameters	Control	Pretreated*	Student's *t*
Plasma area under the curve (nmol ml^{-1} × min)	104 ± 15	188 ± 55	$P < 0.001$
Apparent volume of distribution (ml kg^{-1})	2450 ± 660	1784 ± 285	n.s.
Total plasma clearance (ml min^{-1} kg^{-1})	34·3 ± 4·8	19·8 ± 4·9	$P < 0.001$
Elimination half-life (min)	49·3 ± 10·3	67·9 ± 29·1	n.s.
Free fraction in plasma (% unbound)	17·3 ± 3·1	19·8 ± 3·6	n.s.
Brain concentrations 60**	0·58 ± 0·16	1·31 ± 0·16	$P < 0.001$
(nmol g^{-1} ± s.d.) 180**	0·24 ± 0·14	0·50 ± 0·23	$P < 0.05$

* Rats given denzimol hydrochloride (5 mg kg^{-1} p.o.) 15 min before diazepam (1 mg kg^{-1} i.v.) Results are the mean ± s.d. of 6 control and 5 denzimol-treated rats.

** Minutes after diazepam. Brain concentrations of nordiazepam, *N*-methyloxazepam and oxazepam were below the assay sensitivity limits.

denzimol-treated animals. Elimination as characterized by total plasma clearance (Cl) or half-life ($t_{\frac{1}{2}}$) was impaired by denzimol but only the Cl value reached statistical significance ($P < 0.001$).

Brain concentrations of diazepam were measured at the end of the sampling period (180 min) and, in a separate experiment, 60 min after diazepam injection. At both times diazepam brain concentrations (about double those in plasma) in denzimol-treated rats were about twice those of control animals, the differences being significant (Table 1).

In both control and denzimol-treated animals the plasma and brain concentrations of the active metabolites of diazepam (nordiazepam, *N*-methyloxazepam and oxazepam) were below the assay sensitivity limits.

Denzimol, 2·0 nmol ml^{-1} or approximately the maximum concentrations which would reach the systemic circulation after an oral dose of 5 mg kg^{-1} to rats (Abbiati et al 1986), added in-vitro to plasma containing diazepam, had no effect on the protein binding of the benzodiazepine. Previous studies show that denzimol is unable to displace phenytoin and carbamazepine from their plasma protein binding sites in-vitro even at relatively high concentrations and that the denzimol binding percentage remains constant over a wide range of concentrations (Dal Pozzo et al 1983). The plasma protein binding of diazepam itself was concentration-independent over the concentration range investigated (0·2 and 2·0 nmol ml^{-1}).

Discussion

The metabolism of benzodiazepines and other drugs with a P450-dependent metabolism is impaired if they are administered together with compounds containing aromatic nitrogen bases such as imidazole and related derivatives (Klotz et al 1979; Klotz & Reimann 1980, 1981; Gugler & Jensen 1984; Abernethy et al 1985). This may be related to the capacity of imidazole derivatives to bind P450 and inhibit hepatic mixed function oxidase (Puurunen & Pelkonen 1979; Dickins & Bridges 1982; Hajek et al 1982; Kapetanovic & Kuppferberg 1984; Meredith et al 1985).

Our studies of diazepam indicated that denzimol impairs the elimination of diazepam, a benzodiazepine which is primarily biotransformed by phase I reactions (Schwartz et al 1965). Pretreatment with this imidazole derivative significantly increased (by a factor of about 2) the total plasma area under the curve (AUC) after a single intravenous dose of diazepam consistent with markedly reduced total Cl (about 40%). The V_β value of diazepam was slightly but not significantly reduced by denzimol treatment. The elimination $t_{\frac{1}{2}}$ tended to be prolonged in denzimol-treated rats but the difference did not reach statistical significance; this parameter is influenced by drug distribution according to the well known eqution $t_{\frac{1}{2}} = 0.693 \times V_\beta/Cl$ (Gibaldi & Perrier 1982) and therefore does not exclusively reflect the elimination process. The impairment of diazepam clearance induced by denzimol may be explained by in-vitro findings indicating that denzimol is a potent inhibitor of diazepam *N*-demethylation and C-3 hydroxylation and of carbamazepine epoxidation in the rat (Salmona et al unpublished data).

No nordiazepam, *N*-methyloxazepam or oxazepam could be detected in plasma and brain of control or denzimol-treated rats. This may be because of the small volumes of plasma and brain homogenate we extracted, and the detection limits. However, previous studies have indicated that diazepam active metabolites are present only as traces in rat plasma and brain, suggesting that diazepam alone is presumably responsible for the pharmacological effect after a single dose in rats (Garattini et al 1973; Klotz et al 1976; Caccia et al 1980; Igari et al 1985). These results are typical of the rat because in other animal species the N_1-demethylated and/or C-3-hydroxylated metabolites have been found in plasma and brain in far larger amounts after diazepam injection (Garattini et al 1973).

The finding of brain concentrations of diazepam double those in plasma after injection of the benzodiazepine to control or denzimol-treated rats is consistent with prior studies showing that diazepam concentrates in rat brain and that the time curve of its brain disappearance is almost parallel to that in plasma (Garattini et al 1973; Caccia et al 1980; Igari et al 1982). In fact denzimol treatment approximately doubled diazepam brain concentrations. The implications of this finding cannot be established but since diazepam itself is presumably the main compound responsible for the pharmacological effect after single doses, this rise in diazepam brain concentrations as a probable consequence of its reduced Cl by denzimol might result in an enhancement of diazepam pharmacological activity, under the present experimental conditions. Further studies are needed to evaluate the pharmacological implications of the denzimol-diazepam interaction.

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Inhibition of hepatic microsomal monooxygenase activity by cinchocaine: mechanistic studies and effects of ionization

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The quinoline-based local anaesthetic cinchocaine (dibucaine) was found to be a mixed-type inhibitor of microsomal aminopyrine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase activities from control and phenobarbitone-induced rat liver in-vitro. Cinchocaine also elicited a characteristic type I optical difference spectrum in oxidized liver microsomes (K_s = 24 μM; ΔA_{max} = 3·4 × 10^{-3} absorbance units (nmol cytochrome P450)$^{-1}$) but did not appear to bind to the reduced form of the cytochrome. Additional studies indicated that cinchocaine competitively inhibited the type I spectral binding of substrate (aminopyrine) to ferric cytochrome P450. Studies of monooxygenase inhibition by cinchocaine over a relatively narrow pH range (6·5–8·5) indicated that, as might be expected, the un-ionized form of the drug is associated with inhibitory potency superior to that of the ionized form. Thus 40% inhibition of aminopyrine *N*-demethylase activity was observed with 100 μM cinchocaine at pH 8·0 and 8·5 (24% and 50% un-ionized drug, respectively), whereas only 16% inhibition was observed at pH 6·5 (1% un-ionized drug). These findings suggest that the inhibitory action of cinchocaine is mediated exclusively via an interaction with ferric cytochrome P450 and that the extent of ionization is a determinant of mixed function oxidase inhibition.

A number of studies have demonstrated that nitrogen heterocycles, including imidazoles (Wilkinson et al 1972, 1974a; Murray & Wilkinson 1984), benzimidazoles (Murray et al 1982; Dickins & Bridges 1982; Murray & Ryan 1983) and quinolines (Kahl & Netter 1977; Back et al 1983; Murray 1984), can alter hepatic microsomal xenobiotic metabolism by inhibiting cytochrome P450 (P450)-dependent mixed-function oxidase (MFO) activity. The nature of spectrally observed interactions between inhibitory xenobiotics and P450 has been used as an indication of the mode of MFO inhibition. For example, monoheterocyclic imidazoles generally elicit type II optical difference spectra with ferric P450, a finding that is consistent with a ligand interaction at the haem iron site (Wilkinson et al 1974a, b). On the other hand, fused-ring benzimidazoles substituted in the 2-position with large alkyl or arylalkyl groupings elicit the type I spectral change (Dickins & Bridges 1982; Murray & Ryan 1983). This finding implies that an interaction occurs between the inhibitor and a hydrophobic patch on the P450 apoprotein (Al-Gailany et al 1978). From Hansch analysis the principal determinant of MFO inhibitor potency, in the case of imidazoles and benzimidazoles, appears to be hydrophobicity (Wilkinson et al 1974a; Murray et al 1982), although the importance of steric effects and the position of heteroaromatic substitution has been stressed (Rogerson et al 1977; Murray & Wilkinson 1984).

Hydrophobic interactions have also been hypothesized to be important in the interaction of local anaesthetics with phospholipid membrane bilayers (Papahadjopoulos 1972). Cinchocaine (dibucaine, I) is a potent local anaesthetic of the amide type that is used typically in ointments or, as the hydrochloride, in infiltration anaesthesia (Martindale 1982). The