A RADIORECEPTOR ASSAY TO STUDY THE AFFINITY OF BENZODIAZEPINES AND THEIR RECEPTOR BINDING ACTIVITY IN HUMAN PLASMA INCLUDING THEIR ACTIVE METABOLITES

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- 1 A radioreceptor assay has been established to measure the receptor affinities of numerous benzodiazepines in clinical use.
- 2 The time course of receptor binding activity was studied by this method in the plasma of eight healthy subjects randomly treated with 1 mg lormetazepam (Noctamid®), 2 mg flunitrazepam (Rohypnol®), and 10 mg diazepam (Valium®), and placebo on a cross-over basis. Blood samples were collected up to 154 h after treatment.
- 3 Receptor affinities of numerous benzodiazepines *in vitro* show good correlation with therapeutic human doses ($r = 0.96$) and may be predictive of drug potency in man.
- 4 Mean peak plasma levels of lormetazepam binding equivalents were 4.8 ± 1 ng/ml at 2 h after lormetazepam, 7.2 ± 1.8 ng/ml at 8 h after flunitrazepam, and 17.9 ± 2.7 ng/ml at 15 h after diazepam. Plasma elimination half-lives of benzodiazepine binding equivalents were 9.3, 23 and 63 h, respectively.
- 5 Slow elimination of benzodiazepine binding equivalents following flunitrazepam and diazepam may be due to persistent active metabolites.

Introduction

Radioreceptor assays (RRA) using membrane receptor preparations have been introduced for estimating drug levels in plasma (Creese & Snyder, 1977). These RRA are of great interest because of their ability to detect parent compounds including pharmacologically active metabolites by means of a simple technique. Recently stereospecific and saturable binding sites on cell membranes with high affinity for benzodiazepines (BD) were characterized in the mammalian brain (Squires & Braestrup, 1977; Möhler *et al.*, 1978). As a result of the isolation of these receptors benzodiazepine RRA could be established (Hunt *et al.*, 1978; Lund, 1981). A good correlation between determinations by BD-RRA and single evaluations of parent drug and active metabolites as measured by specific methods was demonstrated (Owen *et al.*, 1979; Lund, 1981). The receptor binding potency of BD *in vitro* correlates well with *in vivo* pharmacological activity as demonstrated for muscle relaxant and anticonvulsive effects (Braestrup & Squires, 1978) as well as anxiolytic activity in animals (Malick & Enna, 1979). Binding equivalents (BE) determined by RRA may also reflect therapeutic activity in man (Braestrup *et al.*, 1977).

The object of this study was to correlate the *in vitro* binding affinities of various benzodiazepines with their recommended clinical doses (Rote Liste, 1980) and to evaluate the time course of elimination for three benzodiazepine hypnotics in man as measured by RRA. Lormetazepam (LMZ), a new potent short-acting BD (Paschelke, 1980), was compared with flunitrazepam (FNZ), a BD of similar potency and elimination rate, which forms active metabolites (Amrein, 1978), and diazepam (DZP), which, as well as its main metabolite desmethyldiazepam (DM-DZP), is eliminated over a long period of time.

Methods

Benzodiazepine radioreceptor assay

BD binding was performed by a modification of the method developed by Lund (1981). Plasma (1 ml) was diluted with 1 ml assay buffer (sodium phosphate, 25 mmol/l, pH 7.4) and extracted with 5 ml ethyl acetate. Depending on BD concentration, 40–1000 μ l of the organic phase were evaporated in duplicate to

dryness in a stream of nitrogen in plastic vials (Eppendorf). The residue was dissolved in 50 μ l buffer.

Lyophilized brain membrane receptor preparation, 400 mg per vial (Ferrosan, Denmark), was suspended in 40 ml assay buffer. Aliquots of 400 μ l were incubated with 50 μ l tracer (0.45 pmol [3 H]-FNZ, 3.1 TBq, New England Nuclear) and either 50 μ l FNZ solution (1 μ mol/l, for unspecific binding), LMZ calibration standards (0.05–5 ng) or plasma extracts. After 30–60 min the test tubes were centrifuged for 5 min at 8000 g. The supernatant was decanted, 300 μ l of a concentrated solution of sucrose was layered onto the pellet to displace residual buffer and the tip of the vial was cut off. The pellet was dissolved in 200 μ l Soluene® (Packard) and the radioactivity determined after additional of 10 ml Picofluor® (Packard).

To estimate the inhibitor affinity constants (K_i) receptor-bound radioactivity was separated by filtration over Whatman FG/B filters followed by three washings with 3 ml cold buffer. BD used were diazepam, desmethyldiazepam, flunitrazepam, flurazepam, nitrazepam, medazepam (Hoffmann-La Roche), temazepam, lorazepam (Wyeth), oxazepam (Thomae) and lormetazepam (Schering).

K_i values were calculated according to the equation.

$$K_i = \frac{IC_{50}}{1 + \frac{C}{K_D}}$$

where IC_{50} is the drug concentration required to

inhibit specific [3 H]-FNZ binding by 50% as read from the logit-log plot of binding data, C is the concentration of the radioactive ligand and K_D its dissoziation constant at equilibrium. Unknown samples were estimated by comparison with a LMZ calibration curve determined in parallel. A correction for recovery was accomplished separately by addition of 10 ng LMZ to 1 ml plasma.

Subjects and procedure

According to a double-blind design eight healthy young volunteers, four women and four men (age 24 to 36 years, mean weight 68 kg), were given single oral doses of 1 mg lormetazepam (Noctamid®, Schering, Berlin), 2 mg flunitrazepam, 10 mg diazepam (Rohypnol®, Valium®, respectively, Hoffmann-La Roche, Basle) or placebo on a cross-over basis. The dose regimen was selected according to the recommendation by the manufacturers (Rote Liste, 1980). Drugs were administered in gelatine capsules at 22.00 h, and heparinized venous blood samples were obtained immediately before and 1, 2, 8, 15, 20, 34, 58, 82 and 154 h after administration. Each drug treatment was followed by a washout period of at least 14 days.

Analysis of data

Treatment effects were evaluated by submitting the RRA data to analysis of variance (three factorial model) followed by Tukey tests. Elimination constants β were determined by analysis of regression of the linearly declining portion of the semi-logarithmic plots (plasma LMZ-BE ν time).

Figure 1 Inhibition of specific [3 H]-FNZ binding by various benzodiazepines. Values represent means of at least three determinations. The dotted lines indicate limits of confidence (20–80 % inhibition).

Figure 2 Correlation of the lowest recommended clinical dose of benzodiazepines with their *in vitro* receptor affinities (K_i), $r = 0.96$.

Results

The concentration dependent inhibition of specific ^3H -FNZ binding by the BD studied, show parallel regression lines in the logit inhibition/log concentration plot (Figure 1), demonstrating comparable ligand-receptor interactions for this class of

substances. The K_i values of these BD were correlated with the lowest recommended clinical dose (Rote Liste, 1980) (Figure 2). Excluding medazepam, which is a prodrug for DZP and DM-DZP (Rieder & Rentsch, 1968) and has a poor affinity to the BD receptor ($K_i > 1000$ nmol/l), a correlation coefficient of $r = 0.96$ was found.

The time course of the mean LMZ-BE in plasma after drug administration is shown in Figure 3. Maximum plasma levels of LMZ-BE as determined in this study were 4.8 ± 1.0 ng/ml 2 h after 1 mg LMZ, 7.2 ± 1.8 ng/ml 8 h after 2 mg FNZ and 17.9 ± 2.7 ng/ml 15 h after 10 mg DZP p.o. Receptor active compounds could not be detected 34 h after LMZ and 82 h after FNZ treatment. In contrast, after DZP administration plasma levels of LMZ-BE (5 ng/ml) were clearly above detection limit up to 154 h. Pre-values as well as plasma values of the placebo treated subjects displayed no RRA activity with exception of one subject on placebo who had been given DZP 2 weeks earlier. The inhibition affinity constants K_i , mean elimination rate constants β , and half-lives $T_{1/2\beta}$ are shown in Table 1. In all individuals receptor active substances were eliminated in the same order: $T_{1/2\beta}$ (LMZ) $< T_{1/2\beta}$ (FNZ) $< T_{1/2\beta}$ (DZP).

Discussion

Relative drug potency has to be determined on the basis of individual dose-response curves. The good correspondence of the K_i values of BD, which

Figure 3 Time course of plasma benzodiazepine binding equivalents after single oral dose of 1 mg lormetazepam (O), 2 mg flunitrazepam (▲) or 10 mg diazepam (●) expressed as ng lormetazepam/ml plasma. Mean of eight volunteers; bars indicate s.e. mean.

Table 1 Kinetic parameters of plasma elimination and receptor affinity of three benzodiazepines and their major active metabolites

Parent drug major active metabolite	K_i (nmol/l)	Elimination rate	Elimination half-life $T_{1/2}$	β (h)
		constant β (h^{-1}) by RRA	by RRA	other methods
Lormetazepam	2.4	$0.075 \pm 0.015^+$	9.3 (5–23)	10 (7–14) ^a
Flunitrazepam	2.4	$0.031 \pm 0.008^{++}$	23 (15–52)	13.5 (9–25) ^b
7-Amino-derivative	—	—	—	23 ^c
N-Desmethyl-derivative	—	—	—	31 ^c
Diazepam	9.3	0.011 ± 0.003	63 (34–180)	32 (21–46) ^d
N-Desmethyldiazepam	13	—	—	(50–99) ^d

+ $P < 0.01$ v FNZ and DZP++ $P < 0.1$ v DZPa Hümpel *et al.* (1980)b Boxenbaum *et al.* (1978)

c Wendt (1976)

d Kaplan *et al.* (1973)

Tukey-test

quantify receptor affinity, to the lowest recommended clinical dose, however, may indicate that RRA data allow an estimate of drug potency in man. Even medazepam as a prodrug with low receptor binding potency conforms to the correlation, considering that the recommended dose is similar to that of DZP and DM-DZP.

BD-BE values obtained by RRA are composite measures of what might be termed the total BD receptor binding potential contained in a given biological sample. This BD binding potential results from both the concentration and receptor affinity of a number of individual BD components which bind to the BD receptor, i.e., parent drug and active metabolites. As different binding affinities of BD result in a parallel shift of the inhibition curves (Figure 1), unknown active BD metabolites should behave similarly in the RRA and be measurable by this method.

Among the BD tested, LMZ appears to have one of the highest affinities to the BD receptor *in vitro*, corresponding well with the low dose effective in clinical practice (Figure 2). The close correspondence of LMZ elimination half-life values resulting from RRA (9.3 h) and specific radioimmunoassay measurements (10 h) confirms the lack of active metabolites (Hümpel *et al.*, 1979; Hümpel *et al.*, 1980). FNZ with a similar K_i as LMZ shows an elimination half-life of 23 h in the RRA which is at variance with pharmacokinetic data reporting a mean plasma half-life of 13.5 h after a single oral dose of FNZ (Boxenbaum *et al.*, 1978). Two major metabolites of FNZ have been described in man (Wendt, 1976; Wickström *et al.*, 1980), the 7-amino derivative and N-desmethyl-flunitrazepam with elimination half-lives of 23 and 31 h, respectively (Wendt, 1976). Both

compounds have been shown to possess pharmacological activity in animals (Amrein, 1978). For N-desmethyl-flunitrazepam 30% of FNZ receptor affinity was reported (Aaltonen *et al.*, 1979). To what extent the 7-amino metabolite contributes to the total BD receptor binding potential in plasma of FNZ-treated subjects is open to question. The apparent discrepancy between plasma elimination rates and half-lives of BD receptor active material as measured by RRA and pharmacokinetic data for FNZ reported by others (Amrein, 1978; Boxenbaum *et al.*, 1978) may be largely due to the additional binding potential of active metabolites. DM-DZP, the major metabolite of DZP, is known to have a longer half-life (50–99 h) than the parent drug itself (32 h) (Kaplan *et al.*, 1973). DM-DZP has about 70% of the receptor affinity of DZP (Table 1).

Again, after DZP, the $T_{1/2}\beta$ of 63 h determined by RRA comprises the time course of total binding potential of the BD components with their different half-lives. The differences in elimination rate constants β of LMZ v FNZ and DZP, respectively, were significant, but not those for FNZ v DZP (Table 1). This may be due to the small number of subjects and the great interindividual variance of β .

The significance of BD levels in plasma with regard to clinical effects is not well understood. There are numerous controversial reports on trials correlating plasma levels or half-lives of BD and some of their major metabolites with hangover and other clinical effects (Dasberg *et al.*, 1974; Dasberg, 1975; Bond *et al.*, 1977; Amrein, 1978). As the particularity of the RRA consists in weighting the amount as well as the receptor affinity of BD including active metabolites, the BD binding potential in plasma should be a more relevant basis for correlations with treatment effects

than single measurements of parent drug or metabolites. Recently, Creese and Snyder developed a RRA for neuroleptics and reported on a good correlation between BE levels in plasma and clinical states (Creese & Snyder, 1977; Tune *et al.*, 1980).

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