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Effect of the triazolobenzodiazepine estazolam on hepatic drug-metabolizing enzyme activity in rats

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Oral doses of the sedative/hypnotic estazolam (500 mg kg^{-1} day^{-1}) to rats for 21 days caused statistically significant increases in liver weight, ascorbate excretion, cytochrome P-450 concentrations, and in aniline hydroxylase, ethylmorphine *N*-demethylase and glutathione *S*-transferase activities, as did approximately equivalent doses of flurazepam hydrochloride. Histologically, the centrilobular hepatocytes were enlarged. Some of these parameters were also increased after doses of estazolam of 100 mg kg^{-1} day^{-1}, but not after 5 mg kg^{-1} day^{-1}, which is about 50-fold greater than a clinical dose. Estazolam was a much less potent enzyme inducer than phenobarbitone under the conditions of these studies.

Benzodiazepines are among the most widely prescribed drugs, usually as sedatives/tranquillizers, but newer benzodiazepines have also been developed as hypnotics with a view to providing a safer replacement for barbiturates. The enzyme-inducing properties of the latter class of compounds is well known (Valerino et al 1974; Ioannides & Parke 1975; for reviews, see Conney 1967; Sher 1971; Remmer 1972; Hunter & Chasseaud 1976; Parke 1979), whereas the benzodiazepines are not generally regarded as potent enzyme-inducing agents. However, diazepam has been claimed to induce microsomal drug-metabolizing enzymes in animals (Heubel & Frank 1970; Valerino et al 1973) and in man (Kanto et al 1974; Sellmann et al 1975), and other examples have been reported of the enzyme-inducing effects of relatively large doses of benzodiazepines in animals (Hoogland et al 1966; Vesell et al 1972; Breckenridge & Orme 1973; Jablonska et al 1975; Fukazawa et al 1975).

This paper reports studies of the enzyme-inducing capability of a newer hypnotic triazolobenzodiazepine, estazolam, in rats in comparison with that of two other benzodiazepines, flurazepam and nitrazepam. Phenobarbitone was used as a reference standard for hepatic enzyme induction.

MATERIALS AND METHODS

Materials

Estazolam (8-chloro-6-phenyl-4H-*s*-triazolo[4,3a]-1,4-benzodiazepine was kindly provided by Takeda Chemical Industries Ltd, Osaka, Japan, flurazepam hydrochloride and nitrazepam by Hoffmann-La Roche & Co Ltd, Basel, Switzerland. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were obtained from the Boehringer Corporation, London, U.K., glutathione (GSH) was from Schwarz-Mann, Orangeburg, N.Y., U.S.A., ethylmorphine hydrochloride B.P.C. from MacFarlan Smith Ltd, Greenford, U.K., and phenobarbitone B.P. from May and Baker, Dagenham, U.K. Ethacrynic acid was a gift from Merck, Sharp and Dohme Research Laboratories, Hoddesdon, Herts., U.K.

Animal experiments

Male CFHB (Wistar-derived strain) rats, ca 150 *g* (Anglia Laboratory Animals, Huntingdon, U.K.), had free access to food (Standard Laboratory Diet No. 1, Spratts, Barking U.K.) and tap water. After acclimatization for 4 days, the animals were allocated randomly into a control and six test groups (8 rats per group). The rats of the test groups received either estazolam (5, 100 or 500 mg kg^{-1} day^{-1}), flurazepam hydrochloride (658 mg kg^{-1} day^{-1}), nitrazepam (95 mg kg^{-1} day^{-1}) or phenobarbitone (50 mg kg^{-1} day^{-1}) respectively by oral intubation for 21 consecutive days. The test compounds were suspended in aqueous 5% (w/v) acacia which was also given to the control animals. The doses chosen were of similar magnitude to those that would be selected for sub-acute or chronic toxicity studies of estazolam in this species. The dose of flurazepam hydrochloride was approximately the molar equivalent of the highest dose of estazolam used but a similar dose of nitrazepam resulted in mortality within 3 days; therefore the nitrazepam dose chosen was approximately the molar equivalent of the intermediate estazolam dose used.

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After the last daily dose, each rat was housed separately in a glass metabolism cage (Jencons Ltd, Hemel Hempstead, U.K.) for collection of 24 h urine for determination of ascorbate excretion (Roe & Keuther 1943).

At 24 h after the last dose, on completion of urine collection, the rats were weighed and killed by cervical dislocation. Their livers were removed immediately, weighed and portions (ca 500 mg) retained for histology. The remainder was homogenized in 50 mM Tris buffer (pH 7·4) containing 0·25 M sucrose (4 vol) at 4 °C using an electrically-driven Potter-Elvehjem-type homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 10 000 *g* for 20 min at 4 °C and the resulting supernatant decanted. This was centrifuged at 105 000 *g* for 1 h at 4 °C (MSE Superspeed 65 Ultracentrifuge) to prepare a microsomal pellet. The 'soluble' post 105 000 *g* supernatant which was dialysed (Boyland & Chasseaud 1969), was stored at −20 °C until assayed for enzyme activity. The microsomal pellet was suspended in 50 mM Tris buffer (pH 7·4) using a hand-operated Potter-Elvehjem type homogenizer, so that 1 ml of the resulting suspension was equivalent to about 300 mg liver (wet weight). This suspension was kept at 4 °C and used within 2 h of preparation.

Drug-metabolizing enzyme activity

Cytochrome P-450 concentrations (Omura & Sato 1967), aniline hydroxylase (Wills 1969), ethylmorphine *N*-demethylase (Cochin & Axelrod 1959) were measured as indicators of microsomal enzyme activity, and GSH S-transferase (using ethacrynic acid as the second substrate; Habig et al 1974), as an indicator of soluble enzyme activity. Protein concentrations were measured by the method of Lowry et al (1951) using bovine serum albumin as a standard.

Statistical analysis

To stabilize variances in data, a logarithmic transformation was performed on some results as indicated in the Tables. A one-way analysis of variance was carried out, and treatment group means were compared with those of the control group using a stepwise version of Dunnett's test (Dunnett 1955, 1964).

Due to heterogeneity of variances, results of glutathione *S*-transferase activity and cytosol protein concentrations were subjected to a non-parametric one-way analysis of variance (Kruskal & Wallis 1952, 1953).

RESULTS AND DISCUSSION

The bodyweight gain of rats dosed with 500 mg estazolam kg^{-1} day^{-1} or with 95 mg nitrazepam kg^{-1} day^{-1}, but not after other treatments, was significantly reduced compared with controls, particularly during the first 7 days of treatment, when presumably the rats were still adapting to these high levels of drug treatment.

The 24 h excretion of ascorbate was significantly increased compared with controls, in all treatment groups except that dosed with 5 mg estazolam kg^{-1} day^{-1}. Urine output was also significantly increased, compared with controls, after all dose levels of estazolam and the other two benzodiazepines (Table 1).

The liver weights of rats receiving estazolam (500 mg kg^{-1} day^{-1}) or flurazepam hydrochloride (658 mg kg^{-1} day^{-1}) were significantly increased compared with controls (Table 1). Histologically, these livers showed enlargement of the centrilobular hepatocytes. In this connection, liver enlargement occurring after phenobarbitone treatment is accompanied by proliferation of the endoplasmic reticulum, particularly the smooth-surfaced membranes, and this is more pronounced in the centrilobular than

Table 1. Daily urine output, ascorbate excretion, liverweight : bodyweight ratios and hepatic protein concentrations (s.d.) in rats dosed orally with estazolam, flurazepam hydrochloride, nitrazepam or phenobarbitone for 21 days.

Treatment (mg kg^{-1})	Urine volume (ml)†	Ascorbate excretion (mg kg^{-1})†	Liverweight : bodyweight ratio (g/100 g)	Microsomal protein (mg g^{-1} liver)	Cytosol protein (mg g^{-1} liver)
Control	15·4 (4·3)	2·1 (0·8)	3·0 (0·2)	16·8 (4·2)	72·8 (16·1)
Phenobarbitone (50)	22·8 (5·6)	18·1 (4·9)**	3·9 (0·2)**	33·2 (2·3)**	68·0 (5·0)
Estazolam (5)	28·2 (10·9)*	3·1 (1·4)	2·9 (0·2)	17·8 (3·4)	72·1 (11·5)
Estazolam (100)	37·1 (16·0)**	11·2 (4·1)**	3·1 (0·2)	22·6 (4·1)*	69·6 (7·9)
Estazolam (500)	54·9 (38·1)**	19·2 (8·6)**	3·9 (0·4)**	30·6 (7·6)**	67·6 (3·0)
Flurazepam HCl (658)	61·3 (24·6)**	34·7 (7·0)**	5·7 (0·4)**	36·5 (5·0)**	56·7 (4·2)*
Nitrazepam (95)	39·1 (24·7)**	4·2 (1·4)**	2·9 (0·1)	16·1 (2·4)	66·2 (9·6)

Significance level, test vs control * $P < 0.05$, ** $P < 0.01$.
† Data logarithmically transformed before statistical analysis.

in the perilobular region (Weibel & Paumgartner 1979). It appears that diazepam treatment can cause proliferation of the smooth endoplasmic reticulum in man (Jezequel et al 1974). However, the livers of rats given the lower dose levels of estazolam were histologically indistinguishable from those of controls. As expected, in those rats showing significant increase in liver weight, hepatic microsomal protein concentrations were also significantly increased, but hepatic cytosol concentrations were unaffected except in those animals treated with flurazepam hydrochloride (Table 1).

Hepatic microsomal enzyme activity was significantly increased after treatment of rats with estazolam at 100, 500 mg kg^{-1} day^{-1}, or flurazepam hydrochloride compared with controls, whereas GSH *S*-transferase activity towards ethacrynic acid was significantly increased after flurazepam hydrochloride, nitrazepam and the highest dose (500 mg kg^{-1} day^{-1}) of estazolam only (Table 2). Tanayama et al (1974) have also shown that estazolam given to rats at 100 mg kg^{-1} day^{-1} for 5 days caused induction of aniline hydroxylase and aminopyrine *N*-demethylase activities and reduction of zoxazolamine paralysis times. However, estazolam did not appear to induce its own metabolism although the duration of its muscle relaxant action, like that of other benzodiazepines, was shortened (Tanayama et al 1974).

Table 2. Hepatic enzyme activities (s.d.) in rats dosed orally with estazolam, flurazepam hydrochloride, nitrazepam or phenobarbitone for 21 days.

Treatment (dose mg kg^{-1})	Cytochrome P-450 (nmol g^{-1} liver)†	Aniline hydroxylase (μmol product g^{-1} liver h^{-1})	Ethylmorphine *N*-demethylase (μmol product g^{-1} liver h^{-1})†	Glutathione *S*-transferase (mmol g^{-1} liver h^{-1})†
Control	5·4 (1·5)	0·92 (0·32)	8·4 (1·7)	0·15 (0·04)
Phenobarbitone (50)	25·9 (4·9)**	2·22 (0·42)**	25·7 (7·5)**	0·23 (0·03)**
Estazolam (5)	6·1 (1·4)	0·98 (0·24)	7·9 (2·8)	0·16 (0·03)
Estazolam (100)	10·6 (3·6)**	1·58 (0·37)**	13·0 (5·0)*	0·22 (0·04)
Estazolam (500)	17·0 (4·9)**	1·85 (0·31)**	18·8 (5·9)**	0·25 (0·02)**
Flurazepam HCl (658)	24·0 (8·0)**	1·60 (0·29)**	29·6 (4·2)**	0·25 (0·08)**
Nitrazepam (95)	5·8 (0·9)	1·06 (0·08)	7·6 (1·3)	0·23 (0·04)*

Significance level, test vs control * $P < 0.05$, ** $P < 0.01$.

† Data logarithmically transformed before statistical analysis.

Induction of hepatic microsomal enzyme activities (Table 2) by phenobarbitone is well-known (Parke 1975). Phenobarbitone is known to also induce GSH *S*-transferase activity in rodent liver (Chasseaud 1979), but induction of these enzymes by benzodiazepines does not seem to have been reported previously.

The results indicated that relatively high doses of estazolam caused liver enlargement and induction of hepatic drug-metabolising enzymes in rats. The extent of this liver enlargement and enzyme induction was somewhat less than that produced by flurazepam (Tables 1, 2) or similar to that produced by chlordiazepoxide and nitrazepam (Tanayama et al 1974), and when reconciled with the dose levels used, it was much less than that caused by phenobarbitone (Tables 1, 2). In the present studies, nitrazepam probably caused less induction of enzyme activity than estazolam at the dose level employed. However, nitrazepam was more toxic than estazolam of which at least five-fold greater doses could be administered to the animals. The enzyme-inducing effects of estazolam appeared to be dose-related, but it would seem unlikely that clinically-used doses of the drug (ca 0·1 mg kg^{-1}, Isozaki et al 1976) would lead to induction of hepatic drug-metabolizing enzymes or liver enlargement in humans. Nevertheless, the induction of hepatic drug-metabolizing enzymes that can occur after large doses of benzodiazepines, while probably irrelevant when extrapolated to the clinical use of these drugs, could obscure the interpretation of the findings of toxicity studies of these drugs, since these large doses are commonly employed in such studies.

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