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EFFECT OF PHENOBARBITAL ON THE *IN VITRO* METABOLISM OF DIAZEPAM IN SEVERAL ANIMAL SPECIES

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Abstract—The metabolic pathway by which diazepam is transformed *in vitro* by liver microsomes from control and phenobarbital-treated rats, mice and guinea pigs was studied in detail.

The increased diazepam metabolism resulting from phenobarbital treatment led to the formation of increased amounts of hydroxylated metabolites in mice, and of both hydroxylated and *N*-demethylated metabolites in rats. In guinea pigs pretreatment with phenobarbital produced only an increased formation of *N*-demethylated metabolite. In all the animal species considered, in addition to the increased diazepam metabolism, phenobarbital treatment led to a decreased recovery of diazepam from incubation medium.

PREVIOUS studies[1] have shown that diazepam is metabolized by isolated perfused liver of rats and mice mostly by two routes, namely *N*-demethylation and hydroxylation in position C_3.

Evidence has also been presented[2] that in conditions of initial steady state velocity the mouse liver microsomes metabolize diazepam predominantly with the formation of *N*-demethyldiazepam, while rat liver microsomes form mainly *N*-methyloxazepam. In addition, *in vitro* studies have shown that *N*-methyloxazepam is demethylated by mouse but not rat liver microsomes, while *N*-demethyldiazepam is hydroxylated to form oxazepam by both species although at a relatively low rate.

It has been amply demonstrated that the drug metabolizing activity present in liver microsomes of several animal species can be altered by the administration of many chemicals. Among the chemical compounds known to stimulate (induce) drug metabolism, phenobarbital has been found particularly effective.[3, 4] The subject of this report is an investigation of the effect of phenobarbital on the metabolism of diazepam by liver microsomes of rat, mouse and guinea pig.

MATERIALS AND METHODS

Male Sprague–Dawley rats (body weight 200–250 g); male albino Swiss mice (body weight 20–25 g) and male albino guinea pigs (body weight 300–350 g) were used. Treated animals were given 40 mg/kg i.p. twice a day of phenobarbital in 0·9% NaCl for 4 days, while control animals received an equivalent volume of 0·9% NaCl. After the last injection, the animals were deprived of food for 24 hr and then sacrificed by decapitation. The livers were removed, weighed, chilled in ice and homogenized in ice

cold 1·15% KCl solution (1:4 w/v). The homogenate was centrifuged at 9000 *g* for 20 min and then the supernatant fraction was again centrifuged at 105,000 *g* for 1 hr (rotor 40′ -Beckman Model L ultracentrifuge). The liver microsomes were suspended in 1·15% KCl solution. Protein determinations were performed according to Lowry *et al.*[5]

Incubation in vitro

An incubation mixture similar to that used by Kato *et al.*[6] consisted of 2·5 ml of microsomal solution equivalent to 1 g of liver; NADP (1·5 μmoles); glucose-6-phosphate (50 μmoles); glucose-6-phosphate dehydrogenase (0·5 units); magnesium chloride (25 μmoles); nicotinamide (50 μmoles); 1·4 ml of 0·2 M phosphate buffer pH 7·4; various concentrations of diazepam (ranging from 10 to 500 μg); 0·45 ml of 1·15% KCl and water to obtain a final volume of 5 ml. The mixtures were incubated in a Dubnoff metabolic shaker at 37° under air for 10 min.

Determination of diazepam metabolites

At the end of the incubation time, the 5 ml of mixture were extracted twice with 10 ml of fresh peroxide-free ethyl ether. The combined ether extracts were taken to dryness, redissolved in a suitable amount of acetonitrile and then gas-chromatographed as previously described.[7] The gas-chromatographic technique used allows measurement of both the amount of metabolites formed and the disappearance of the substrates added. Recovery studies of diazepam and its metabolites from water and from microsome incubation mixture were reported elsewhere.[2]

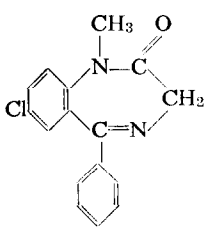

FIG. 1.

RESULTS

Mouse liver microsomes

Results of experiments in which scalar amounts of diazepam were incubated with liver microsomes from control and phenobarbital treated mice, indicated that diazepam was metabolized into three compounds: *N*-methyloxazepam, *N*-demethyl-diazepam and oxazepam. The effect of phenobarbital treatment of mice on diazepam metabolization is shown in Table 1. Incubation with control mice liver microsomes in conditions of V_{max} (200 μg of diazepam added) led to the disappearance of 26 per cent of the substrate.

About 14 per cent was metabolized to *N*-methyl-oxazepam, about 11 per cent to *N*-demethyldiazepam and only a trace amount to oxazepam. The stimulation of the metabolism of diazepam induced by phenobarbital is evident from the finding that, in the same experimental conditions, 76·4 per cent of diazepam added disappeared after incubation. This significant increase in diazepam metabolism was accompanied

TABLE 1. METABOLISM *IN VITRO* OF DIAZEPAM BY LIVER MICROSOMES OF CONTROL AND PHENOBARBITAL TREATED MICE

Mouse	Diazepam added (μg)	Substrate and metabolites recovered after 10 min incubation: Diazepam (μg)	*N*-methyl-oxazepam (μg)	*N*-demethyl-diazepam (μg)	Oxazepam (μg)
Controls	10	3·30 ± 0·001	n.d.	5·75 ± 0·002	n.d.
	20	7·91 ± 0·001	n.d.	11·12 ± 0·001	n.d.
	30	14·00 ± 0·02	n.d.	15·10 ± 0·04	n.d.
	40	18·30 ± 0·01	2·10 ± 0·001	17·62 ± 0·01	n.d.
	60	32·00 ± 0·02	7·50 ± 0·01	19·20 ± 0·01	n.d.
	75	39·10 ± 0·01	10·40 ± 0·22	20·92 ± 0·11	n.d.
	100	57·30 ± 0·01	15·50 ± 0·04	22·22 ± 0·05	n.d.
	150	103·08 ± 0·02	21·60 ± 0·60	22·00 ± 0·05	n.d.
	200	148·56 ± 0·02	28·37 ± 0·001	19·10 ± 0·02	n.d.
Phenobarbital-treated	10	n.d.	n.d.	3·90 ± 0·01	1·60 ± 0·01
	20	n.d.	n.d.	8·00 ± 0·02	2·50 ± 0·02
	30	n.d.	n.d.	12·00 ± 0·01	2·95 ± 0·01
	40	3·50 ± 0·02	1·60 ± 0·02	12·49 ± 0·02	3·00 ± 0·02
	60	3·90 ± 0·01	8·10 ± 0·001	17·90 ± 0·33	2·90 ± 0·02
	75	18·40 ± 0·10	14·40 ± 0·03	18·40 ± 0·03	2·82 ± 0·10
	100	18·50 ± 0·01	26·30 ± 0·12	18·50 ± 0·12	2·70 ± 0·02
	150	33·60 ± 0·01	39·10 ± 0·10	18·00 ± 0·10	2·80 ± 0·10
	200	47·20 ± 0·33	52·00 ± 0·02	18·90 ± 0·12	3·10 ± 0·10

n.d. < 0·05 μg.

TABLE 2. METABOLISM *IN VITRO* OF DIAZEPAM BY LIVER MICROSOMES OF CONTROL AND PHENOBARBITAL TREATED RATS

Rat	Diazepam added (μg)	Substrate and metabolites recovered after 10 min incubation: Diazepam (μg)	*N*-methyloxazepam (μg)	*N*-demethyldiazepam (μg)
Controls	10	6·91 ± 0·08	2·20 ± 0·01	n.d.
	20	14·50 ± 0·05	4·10 ± 0·02	0·52 ± 0·01
	30	21·85 ± 0·03	5·60 ± 0·02	0·79 ± 0·01
	40	30·40 ± 0·03	6·80 ± 0·01	1·05 ± 0·02
	60	47·32 ± 0·01	9·31 ± 0·10	1·35 ± 0·02
	100	82·50 ± 0·01	14·00 ± 0·08	1·97 ± 0·01
	200	166·60 ± 0·02	25·62 ± 0·05	3·92 ± 0·03
	300	253·12 ± 0·10	37·12 ± 0·10	5·57 ± 0·02
	500	461·12 ± 0·08	35·00 ± 0·12	5·00 ± 0·02
Phenobarbital-treated	10	n.d.	n.d.	n.d.
	20	n.d.	n.d.	n.d.
	30	1·80 ± 0·05	7·40 ± 0·08	2·00 ± 0·01
	40	3·12 ± 0·03	12·81 ± 0·05	4·40 ± 0·02
	60	6·24 ± 0·03	18·20 ± 0·03	5·40 ± 0·02
	100	16·21 ± 0·10	34·41 ± 0·03	8·61 ± 0·03
	200	64·73 ± 0·12	80·70 ± 0·02	10·00 ± 0·03
	300	132·41 ± 0·12	118·00 ± 0·12	10·10 ± 0·01
	500	304·60 ± 0·05	139·40 ± 0·10	10·00 ± 0·03

* n.d. < 0·05 μg.

by a marked increase in the formation of *N*-methyloxazepam (97·7% increase) and oxazepam, which was present only in trace amounts when liver microsomes of control mice were employed, while the amounts of *N*-demethyldiazepam did not significantly change. It is evident, therefore, that phenobarbital treatment resulted in an increased hydroxylation of diazepam to form *N*-methyloxazepam and oxazepam, while the *N*-demethylation step was apparently unaffected.

It is of interest to note that over 90 per cent of diazepam incubated was recovered, partly as such, partly as metabolized products, with liver microsomes from control mice. In contrast to these results benzodiazepines recovered, after incubation of diazepam with liver microsomes from phenobarbital treated mice, was only 60 per cent of the incubated diazepam (100 μg).

Rat liver microsomes

Results of experiments in which scalar amounts of diazepam were incubated with liver microsomes from control and phenobarbital-treated rats, are reported in Table 2. Incubation with control rats liver microsomes in conditions of V_{max} (300 μg of diazepam added) led to the disappearance of about 15 per cent of the substrate.

Nearly 12 per cent was metabolized to *N*-methyloxazepam and only a small amount (1·8%) to *N*-demethyldiazepam. No oxazepam was found in this experimental condition.

Also with liver microsomes from phenobarbital treated rats the stimulation of diazepam metabolism is evident. Both the metabolic pathways of diazepam were enhanced but to different extents. The *N*-demethylation reaction was doubled while

TABLE 3. METABOLISM *IN VITRO* OF DIAZEPAM BY LIVER MICROSOMES OF CONTROL AND PHENOBARBITAL TREATED GUINEA PIGS

Guinea pig	Diazepam added (μg)	Substrate and metabolites recovered after 10 min incubation*	
		Diazepam (μg)	*N*-demethyldiazepam (μg)
Controls	10	n.d.	8·92 ± 0·12
	20	n.d.	17·91 ± 0·08
	30	10·80 ± 0·03	19·00 ± 0·03
	40	17·90 ± 0·02	21·80 ± 0·02
	60	21·70 ± 0·02	35·55 ± 0·10
	100	38·21 ± 0·01	50·90 ± 0·12
	150	69·53 ± 0·02	69·30 ± 0·03
	200	112·60 ± 0·01	77·40 ± 0·10
	300	215·10 ± 0·02	84·22 ± 0·10
	500	419·30 ± 0·12	80·33 ± 0·12
Phenobarbital-treated	10	—	7·55 ± 0·03
	20	—	12·71 ± 0·02
	30	—	24·00 ± 0·10
	40	—	31·95 ± 0·08
	60	—	39·71 ± 0·06
	100	—	75·30 ± 0·03
	150	—	110·15 ± 0·12
	200	—	125·72 ± 0·08
	300	—	180·60 ± 0·10
	500	2·80 ± 0·12	250·00 ± 0·10

* n.d. < 0·05 μg.

the C_3 hydroxylation was 4-fold higher. No oxazepam was found even after phenobarbital induction.

Guinea pig liver microsomes

Table 3 shows that the metabolism of diazepam by control guinea pig liver microsomes was qualitatively and quantitatively different to that obtained in the experiments with control mouse and rat liver microsomes. In fact no hydroxylation of the substrate occurred, while the major metabolic pathway of diazepam was the *N*-demethylation.

The increased diazepam metabolism resulting from phenobarbital treatment again led to the formation of increased amounts of *N*-demethyldiazepam.

In addition, the failure to extract diazepam completely was particularly evident using liver microsomes from phenobarbital-treated guinea-pigs (no diazepam was recovered when 10 to 300 μg was added to the incubation medium).

TABLE 4. EFFECT OF PHENOBARBITAL ON DIAZEPAM METABOLISM BY LIVER MICROSOMES IN DIFFERENT ANIMAL SPECIES

	Increase by phenobarbital induction	
Species	Hydroxylation formation of *N*-methyloxazepam (%)	*N*-demethylation formation of *N*-demethyldiazepam (%)
Mouse	109	0
Rat	233	323
Guinea pig	0	426

Table 4 is a summary of the observed differences in diazepam metabolism after administration of phenobarbital to several animal species. The results are quite striking, because in the experimental conditions used phenobarbital increases hydroxylation in mice, *N*-demethylation in guinea pigs and both metabolic pathways in rats.

DISCUSSION

The findings reported in this paper show how an interaction between two drugs may lead to different results depending on the animal species in which the interaction is evaluated. Phenobarbital, an agent known to increase the reticulum endoplasmic system of the liver and therefore the enzymatic activity present in the membranes of this system,[3, 4] affects the metabolism of diazepam. In the mouse where diazepam is predominantly metabolized to form *N*-demethyldiazepam,[12] phenobarbital increases mostly the hydroxylative pathway and therefore there is an increase in the formation of *N*-methyloxazepam and oxazepam.

In the guinea pig where diazepam is only *N*-demethylated, phenobarbital markedly increases only demethylation without affecting the hydroxylation pathway, judging by the formation of *N*-methyloxazepam and oxazepam.

In the rat where diazepam is mainly metabolized to form *N*-methyloxazepam, phenobarbital increased both *N*-demethylation and hydroxylation processes.

There are however two limitations in the present study. Firstly, phenobarbital was

given to the three animal species at the same dose and this may not be the optimal condition for induction for all species considered. Secondly the relative increase of hydroxylation or *N*-demethylation induced by phenobarbital is assessed by the formation of the relative metabolites.

However while in control animals the disappearance of diazepam is completely accounted for by the formation of the metabolites, in induced animals (phenobarbital treated) the metabolites formed represent only a fraction of the diazepam apparently metabolized. The hypothesis that diazepam not recovered as metabolites could have been bound to some constituents of the reticulum endoplasmic system proliferating under phenobarbital treatment[9–11] did not receive experimental support in our attempts to recover more diazepam by changing the conditions of the extraction.

It may be possible instead that minor routes of diazepam metabolism become important under the action of phenobarbital. It may also be possible that in induced animals the diazepam metabolites can be further metabolized to form compounds not measurable in our experimental conditions. In this respect it is interesting that Schwartz *et al.*[8] have very recently suggested that phenobarbital treatment in rats may cause an increase of more polar and non-ether extractable diazepam metabolites.

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