Comparison of dihydrodiazepam enantiomers: metabolism, serum binding and brain receptor binding

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Summary. Dihydrodiazepam is a diazepam prodrug, as shown by its in vitro metabolism by rat and mouse liver and brain microsomal fractions, and its displacing activity on brain diazepam binding. The mechanism of bioactivation is discussed. Stereoselectivity of metabolism and of binding to specific benzodiazepine binding sites in brain synpatosomes and serum albumin were studied.

The study of 1,4-benzodiazepines has revealed several stereoselective processes, which are all connected with chirality in position 3 of the diazepine ring system[2-9]. 4,5-Dihydrodiazepam (DHD) seems to be a suitable compound for studying the influence of another part of the ring system on stereoselective pharmacokinetic processes. (−)-DHD was shown[10] to have about 2-3 times stronger anticonvulsant and antiaggressive effects in mice than its antipode. The in vivo metabolism of (−)-DHD in the rat was shown[11] to proceed via N_1-demethylation and through the formation of diazepam (D) and its further metabolism. We have investigated in vitro the mechanism of the N_4-C_5 oxidation and other pharmacokinetic processes which might contribute to the different pharmacological effects of the enantiomers.

Materials and methods. Synthesis and resolution of DHD and its 2-^{14}C-labelled form were carried out as described[12,13]. The specific activity was 6.0 mCi/mmole, the radiochemical purity was 98.9%. Brain and liver microsomal fractions of mice and rats were prepared by a common procedure. The 13,000 × g, 15 min supernatants of liver and brain homogenates (in 0.25 and 0.32 M sucrose, respectively) were centrifuged at 120,000 × g for 60 min. The microsomes were freshly combined with an NADPH-regenerating system[14]. After 10 min of preincubation at 37 °C incubation was performed with DHD for 10 min. Aliquots of the ether extracts were chromatographed on thin-layer plates in 2 directions[11]. Radioactivity of the spots was determined on a liquid scintillation spectrometer.

Labelled DHD was equilibrated between rat or mouse serum diluted 1:1 with 0.05 M phosphate buffer (pH = 7.4) and the same buffer for 3.5 h at 20 °C according to the method of Weder and Bickel[15]. A synaptosomal fraction of whole rat brains was prepared as described[16]. The binding assay was performed with 4.3 nM of 1-^{3}H-D (5.2 Ci/mmole) according to Möhler and Okada[7].

Results and discussion. The in vitro accumulation of 3 main metabolites of DHD also found in vivo[11] was detected: demethyl-DHD (deMe-DHD), D and demethyl-D (deMe-D). No stereoselective differences could be demonstrated in DHD metabolism in the rat liver microsomal fraction (figure). DHD metabolism was faster in liver microsomes from mice (table 1) than from rats (table 2, compare the columns for controls). Similar species differences were found for the N_1-demethylation of benzodiazepines[17]. (−)-DHD led to a significantly higher D concentration in the

Table 1. Metabolism of dihydrodiazepam enantiomers by mouse liver and brain microsomes and the effect of cofactors

		(+)-DHD	(−)-DHD Control	Without NADPH-regen. system	N_2-atm.	SKF 525A (0.5 mM)	CO[a]
		pmole / 10 min × mg protein		% of the average control values			
Liver	D	108 ± 4[d]	143 ± 15	43[b]	52	43[b]	80
	deMe-DHD	24 ± 4[b]	86 ± 8	34[b]	17[b]	22[b]	24[b]
	deMe-D	45 ± 4	32 ± 6	59[d]	78	41[b]	81
Brain	D	26.4 ± 0.8	25.7 ± 3.3	99	52[c]	69	103
	deMe-DHD	3.0 ± 0.6	3.2 ± 1.1	67	67	50[d]	59
	deMe-D	2.7 ± 0.9	2.3 ± 0.7	210[d]	57[d]	63	152[d]

The amounts of metabolites formed after incubation with the enantiomers (1.1 µmole/l) are indicated. Data are averages (± SEM) of 3 determinations. [a]With 20 v% O_2; [b]$p < 0.01$ compared to control (−)-DHD Student's t-test; [c]$p < 0.025$ vs control (−)-DHD; [d]$p < 0.05$ vs control (−)-DHD.

Table 2. The effect of metabolic inhibitors and activators on the metabolism of (−)-DHD by rat liver microsomes

	Control	Boiled micr.	Without NADPH-regen. system	N_2-atm.	SKF 525A (0.5 mM)	Dithio-threitol (1 mM)	N.heptyl amine (2 mM)
	pmole / 10 min × mg protein	% of the control values					
D	31.6 ± 2.7	28[a]	28[a]	45[a]	37[a]	107	72[c]
deMe-DHD	11.9 ± 1.8	13[a]	20[a]	40[b]	28[a]	211	36[b]
deMe-D	6.7 ± 1.9	15[b]	19[c]	45	18[b]	112	22[c]

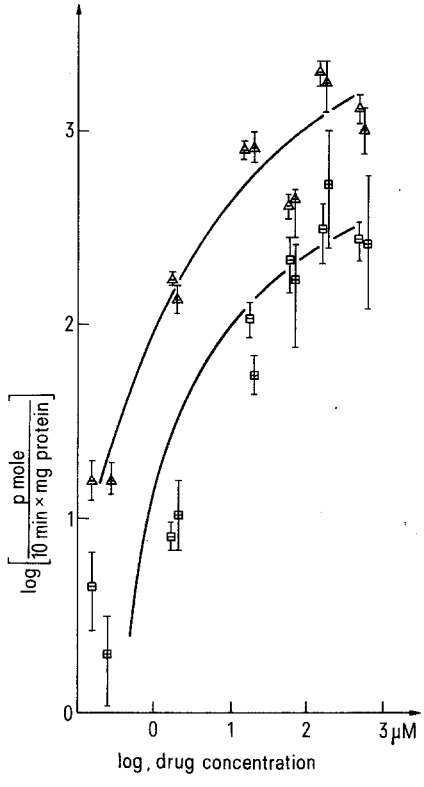
Indicated are the amounts of metabolites after incubation of (−)-DHD (1.8 µmoles/l). Data are averages (± SEM) of 3 determinations. [a]$p < 0.01$ vs control %; [b]$p < 0.025$ vs control %; [c]$p < 0.05$ vs control %.

hepatic microsomes of mice (table 1), than (+)-DHD, which may contribute to its stronger pharmacological activity.

The recent demonstration of the drug metabolizing activity[18] and cytochrome P-450 content[19] of brain microsomes raised the possibility that slight metabolic differences in the brain in situ might contribute to the stereoselectivity of pharmacological activities. Brain microsomes of the mouse did metabolize the antipodes, however without stereoselectivity (table 1). The slower formation of metabolites reflects the smaller metabolizing capacity of brain as compared to liver. This difference is increased by the different yield of microsomes. The effect of cofactors and metabolic effectors was also examined (table 1). The results are compatible with the involvement of cytochrome P-450 in the liver microsomes, but not in brain microsomes. The oxidation of parathion by brain microsomes did not depend on NADPH either[20]. The role of non-cytochrome P-450 oxygenases was suggested in the brain metabolism of aminazin[18].

The formation of D is not inhibited by dithiothreitol nor activated by n.heptyl amine in rat hepatic microsomes (table 2). These observations, and the inhibitory effect of SKF 525A, exclude the possibility that oxidation of the N_4-C_5 bond proceeds via N_4-oxidation[21]. Although 3-hydroxylation is common for benzodiazepines, 3-hydroxylated metabolites of DHD were not found[11]. The idea that these compounds are dehydrated spontaneously is supported by our chemical evidence; catalytic dehydrogenation of oxazepam always led to the formation of deMe-DHD. The rate of 3-hydroxylation of benzodiazepines shows the same species differences[22] as are demonstrated here for D formation from DHD. Thus we suggest that D and deMe-D are formed by the liver microsomes also from the 3-hydroxylated 4,5-dihydro-derivatives after spontaneous dehydration.

Binding characteristics to rat and mouse sera were calculated from Scatchard plots (table 3). The slightly higher binding of the (−)-enantiomer cannot explain the similar stereoselectivity of the pharmacological activities. The enantiomers were equally able to displace ^{3}H-D in the synaptosomal fraction of rat brain (IC_{50} = 360 nM). Hence, differences in the receptor affinities of the antipodes do not seem to contribute to the selective pharmacological activity either. Their displacing activity was weaker than that of nonlabelled D (IC_{50} = 21 nM). In conclusion, DHD has to be considered a prodrug of D. It is suggested that its C_3-hydroxylated metabolites contribute to the formation of D in liver. Bioactivation also proceeds in the brain in situ, and is probably not connected with cytochrome P-450. The basic pharmacodynamic processes show low selectivity for chirality at C_5 and they cannot explain the stereoslelective pharmacological activity of DHD.



The effect of concentration of DHD enantiomers on the rate of formation of D (△ and ▲) and deMe-DHD (⊟ and ⊞) by rat liver microsomes. Points are averages (± SEM) of 4 determinations. Incubation was performed for 10 min at 37 °C.

Table 3. Scatchard analysis of the binding of DHD enantiomers to rat and mouse sera

	K(mM^{-1})		n (µequiv. / g proton)	
	(+) DHD	(−) DHD	(+) DHD	(− DHD
Mouse	22.4	64.2	3.9	1.8
Rat	4.5	6.9	15.0	11.1

Data are determined from 10 experiments representing different substrate concentrations between 1 and 300 µmoles/l.

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