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SHORT COMMUNICATIONS

Postnatal development of sex-dependent differences in the metabolism of diazepam by rat liver

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It has been demonstrated in a number of studies [1–8] that the pharmacokinetics of some drugs exhibit sex-dependent differences. The nature and degree of these differences vary with the species and drug under investigation. The rat has been most extensively studied and sex-related differences have been observed for a variety of substrates [1, 3, 4]. Recent evidence suggests that for certain drugs such differences may also exist in the human [2, 5–8], e.g. MacLeod *et al.* [8] have found that the half-life of diazepam was significantly shorter in men than in women. The reasons for these differences are not known and could be caused either singly or in combination by the hepatic metabolism of diazepam, the excretion of the drug or additional factors.

Since diazepam is extensively metabolized in the liver [9, 10], hepatic biotransformation could in this case play an important role in the sex-related difference observed. Diazepam is transformed *in vivo* [9, 10] and *in vitro* [11–13] in several animal species and in the human by two major pathways, 3-hydroxylation and *N*-demethylation (Scheme 1). To our knowledge no sex-related difference in the relative importance of these two pathways has been reported. As model system, we have therefore studied the metabolism of diazepam by the 6000 *g* supernatant of rat liver homogenate obtained from male and female rats at different ages and in two different strains.

Drugs and internal standards. Diazepam, desmethyldiazepam, 3-hydroxydiazepam and oxazepam were kindly donated by Dr. W. Vetter (Hoffman-La-Roche, Basel, Switzerland), and prazepam by Dr. K.-O. Vollmer Gödecke, Freiburg, G.F.R.).

Chemicals. Ethyl acetate (Nanograde) was obtained from Byk-Mallinckrodt (Wesel, G.F.R.), absolute ethanol from Merck (Darmstadt, G.F.R.), NADPH from Boehringer (Mannheim, G.F.R.), and isocitrate and isocitrate dehydrogenase from Sigma (Munich, G.F.R.).

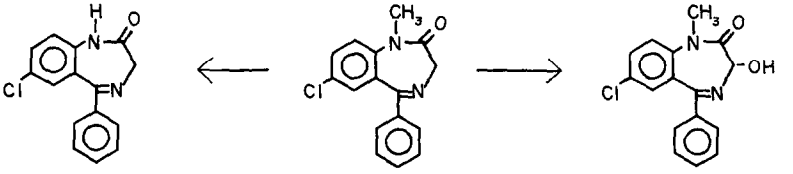
Animals and hepatic preparations. Male and female rats of the Wistar and BD-strains were killed by decapitation and the livers were quickly excised and placed into ice-cold Tris–KCl buffer, pH 7.4 (25 mM Tris, 0.15 M KCl). After chopping the livers and rinsing the fragments, homogenates were prepared by a polytron unit PT-10 (20 sec of sonication) to a final concentration of 20% (w/v) in the same buffer. The 6000 *g* supernatants were prepared by centrifugation for 10 min.

Incubations were carried out at 37° in 1.5 ml Eppendorf Micro-tubes in a shaking water bath under free access to air. The incubation mixture (final volume: 250 μl) contained NADPH (100 μM) and a NADPH-regenerating system (isocitrate, isocitrate dehydrogenase) in Tris–KCl buffer. The appropriate amount of diazepam (final concentration between 1 and 600 μM) was added in 2.5 μl ethanol). This mixture was preincubated at 37° for 5 min and the reaction initiated by the addition of 25 μl of the appropriately diluted hepatic 6000 *g* supernatant. After a reaction time of 12 min, 200 μl of the incubate were transferred into another Eppendorf reaction vessel containing 1 ml ethyl acetate and the internal standard (75 ng prazepam).

Analysis of diazepam metabolites. Extraction was carried out on a reciprocal shaker for 20 min. After a 2 min centrifugation period (5012 Eppendorf centrifuge), the organic supernatant was transferred into a 1.5 ml glass vial and the solvent evaporated to dryness under a stream of nitrogen. The residue was dissolved in 60 μl ethyl acetate and 1 μl was injected into the electron capture gas chromatograph (model 251 ECD on a Carlo-Erba 2300 gas chromatograph). A 2 m glass column which was packed with 3% OV-17 on 120/140 Gas Chrom Q (Applied Science Lab.) was used. The oven temperature was maintained at 290°, the temperatures of the injector and detector were 300°. Argon–5% methane was used as carrier gas. Extraction yields of metabolites were between 80 and 100 per cent, the variability coefficients between 3 and 5 per cent, and the lower limit of detection between 2 and 5 ng/ml (using 200 μl sample volumes). Calibration was achieved by the analysis of samples to which known amounts of metabolites had been added. Linear relationships between peak height ratios and amounts of metabolites added were found over a concentration range of three orders of magnitude.

Gas chromatography–mass spectrometry (GC–MS). The identity of the metabolites present was confirmed by GC–MS-Computer analysis as described earlier [14]. Selected ion monitoring for low-level detection of the benzodiazepine metabolites was also used.

For the *in vitro* measurement of diazepam metabolism, we have chosen to use the 6000 *g* supernatant of liver homogenate instead of the 'microsomal' fraction, since



N-desmethyldiazepam diazepam 3-hydroxydiazepam

Scheme 1

Table 1. Michaelis–Menten constants of the 3-hydroxylation and demethylation reaction of diazepam by rat liver *in vitro** (Wistar strain)

	Reaction products 3-Hydroxydiazepam		Desmethyldiazepam	
	V_{max}§	K_m‖	V_{max}§	K_m‖
Neonatal rat†				
Male	34	240	9.1	110
Female	48	310	10.3	100
Male/female	0.71	0.77	0.88	1.1
Adult rat‡				
Male	697 ± 115	564 ± 95	103 ± 8.5	265 ± 43
Female	135 ± 26	259 ± 30	117 ± 21	608 ± 105
Male/female	5.2	2.2	0.88	0.44

* Supernatant (6000 *g*) prepared as 20% w/v liver in Tris/KCl buffer; total incubation volumes were 250 μl and contained 25 μl of neonatal or 2.5 μl of adult rat liver supernatant. Incubation time: 12 min; sample volume for GC-ECD analysis: 200 μl.

† Ten livers of each gender were pooled. The squares of the linear regression coefficients of the Michaelis–Menten kinetics for the two metabolic reactions measured were: 3-hydroxylation, male ($r^2 = 0.98$), female ($r^2 = 1.00$); demethylation, male ($r^2 = 0.96$), female ($r^2 = 1.00$).

‡ Values given as mean ± S.D. (N = 4).

§ [nmole/g liver/min].

‖[μM].

significant losses of enzyme activity were observed if microsomes were prepared from fetal and neonatal livers [15]. The activities of the enzymes measured were calculated on the basis of liver weight rather than protein content.

For the adult Wistar rat the value of V_{max} for the demethylation of diazepam was slightly lower for the male than for the female, however, for the 3-hydroxylation of diazepam the value of V_{max} for the male exceeded that of the female by a factor of 5.2 (Table 1). The apparent K_m values for the two reactions did not show such a large difference.

The initial rates of the formation of the 3-hydroxydiazepam and *N*-desmethyldiazepam by the 6000 *g* hepatic supernant of another strain of the rat (BD-strain) are shown in Table 2. The *N*-demethylating enzyme activities of the adult male exceeded those of the female by a factor of 2.6. An even larger difference was found for the formation of the 3-hydroxylated product, the adult male enzyme activity exceeded adult female activity by a factor of 10. This is a much larger factor than has been reported up to now for the sex difference of a drug metabolizing enzyme activity.

The enzyme activity for the formation of both 3-hydroxydiazepam and *N*-desmethyldiazepam was found to be much lower in the newborn rat than in the adult rat (Table 1, Wistar rat). This was to be expected since it is well established that in most animal species the drug metabolizing enzyme activities develop postnatally and are at very low levels pre- and perinatally [cf. 16]. In contrast to the adult rats, only very small sex-linked differences for the kinetic parameters of the two diazepam metabolizing reactions were found in neonatal rat liver (Table 1).

Induction and inhibition studies indicate that diazepam is likely to be metabolized by a cytochrome P-450 dependent enzyme system. Thus, while the low content of this enzyme system in neonatal rat liver leads to low diazepam

* Unpublished results from this laboratory.

Table 2. Initial rate of diazepam metabolite formation by adult rat liver *in vitro**† (BD-strain)

	Reaction products‡ 3-Hydroxydiazepam	Desmethyldiazepam
Adult male	74.5 ± 9.6	12.9 ± 2.1
Adult female	7.4 ± 0.90	4.9 ± 0.32
Male/female	10.1	2.6

* Incubation conditions as in Table 1.

† Substrate concentration: 17.6 μM (5 μg/ml).

‡ Values given as [nmole/g liver/min]; mean ± S.D. (N = 10).

metabolism in neonatal rat as compared to adult rat, the sex-related difference in the adult rat cannot be explained by different cytochrome P-450 contents since the level of this enzyme system is similar in adult male and female rat (0.6 and 0.5 nmole/mg microsomal protein, respectively).

A much more likely explanation is that different forms of the cytochrome P-450 dependent monooxygenase system may be responsible for the *N*-demethylation and 3-hydroxylation of diazepam (cf. the kinetic constants shown in Table 1). These enzymes exhibit approximately the same activity at birth. Postnatally, the *N*-demethylating enzyme system develops at similar rates in the male and female rat, while the 3-hydroxylating enzyme system develops to reach far higher activity in the male than in the female rat.

Further evidence for the apparent multiplicity [17, 18] of the cytochrome P-450 system includes: (a) the 3-hydroxylating activity rapidly decreased on repeated thawing and freezing of the liver 6000 *g* supernatant, while the *N*-demethylating activity was only slightly affected, suggesting different stabilities of the two enzyme systems;* (b) pretreatment of the rat with phenobarbital strongly increased both the 3-hydroxylation and *N*-demethylation *in vitro*, while pretreatment with 3-methylcholanthrene decreased the 3-hydroxylation while the *N*-demethylation was increased, albeit less than after phenobarbital pretreatment.*

Because of the ease, specificity and sensitivity of the analytical procedures developed and the magnitude of the observed sex-differences, the *in vitro* hepatic metabolism of diazepam provides an interesting and convenient model for the study of sex-linked differences in drug metabolism.

In conclusion, we have compared the diazepam metabolizing enzyme activities of male and female rat liver. The rate of 3-hydroxylation of diazepam by the adult male rat exceeds that of the adult female rat by a factor of 10 (BD-rat) or 5.2 (Wistar rat). The rate of *N*-demethylation of diazepam showed much smaller differences. For the neonatal rat, neither the 3-hydroxylating nor *N*-demethylating activities showed a sex-dependent difference. Evidence is presented that the two metabolic reactions are catalysed by different forms of the cytochrome P-450 system.

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Trans-membrane alkylation: a new method for studying irreversible binding of reactive metabolites to nucleic acids

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Examination of metabolites capable of alkylating nucleic acids has become an important point in assessment of possibly carcinogenic compounds. Alkylation of nucleic acids by metabolites of carcinogens can be demonstrated *in vivo* [1], but application of such methods is hampered by the necessity of administration of very large quantities of expensive radioactive material [2]. On the other hand, many carcinogens are converted by microsomal monooxygenases *in vitro* to the reactive metabolites which covalently bind to macromolecules [3, 4]. It has been proposed to add a protein, e.g. albumin, to incubations of rat liver microsomes, NADPH, and the labeled compound in question [5]. Re-isolation of the protein component after the incubation allows measurement of irreversible protein binding to a well defined molecule. Similarly, nucleic acids are often added to microsomal incubations to provide targets for alkylation [4, 5]. Besides DNA and RNA, the use of synthetic polynucleotides (polyadenylic acid, polycytidylic acid, etc.) gives invaluable insights into the type of nucleotide preferred for alkylation and into the possible mechanisms [6, 7]. A disadvantage of this method of using natural or artificial nucleic acids as trapping material for radioactive metabolites *in vitro* is the presence of nucleases in microsomal preparations, and the presence of ribosomal RNA in microsomes which often leads to difficulties in estimation, especially of DNA adducts. Also, nucleic acids re-isolated after incubations with microsomes may be contaminated with proteins originating from the incubation system; in general, proteins, according to their content of free sulfhydryl groups, are alkylated by electrophilic metabolites to a much higher degree than are nucleic acids. The presence of nucleases in microsomal incubations usually leads to unsatisfactory recoveries of nucleic acids on re-isolation from such incubations and to degradation of bioactive molecules. Especially if the alkylated re-isolated macromolecules are to be subsequently subjected to analyses using methods of molecular biology, a method is required which excludes direct action of microsomal enzymes on the macromolecule incubated.

This report describes a new method for alkylating nucleic acids by metabolites of xenobiotics in rat liver microsomal incubations which overcomes these difficulties. In this system, microsomal biotransformation and the binding target are separated into two compartments, separated from each other by polyamide molecular sieves. [1,2-^{14}C]Vinyl chloride is used as substrate to demonstrate the practicability of the method; this compound is known [6–8] to be transformed by rat liver microsomes to an alkylating metabolite. As a chemically well-defined target for alkylation, polyadenylic acid (poly-adenosine) is used because it gives a single well characterized alkylation product (1,N^6-ethenoadenosine) on reaction with vinyl chloride metabolites [6, 8]. Results obtained by the conventional method (method A) are compared with those of the new 'trans-membrane-alkylation' method (method B).

Microsomal incubations. Rat liver microsomal incubations were carried out under an atmosphere containing [1,2-^{14}C]vinyl chloride, using the all-glass incubation apparatus previously described [9]. Conditions of substrate saturation were chosen [9], i.e. vinyl chloride in the gas phase exceeded a partial pressure of 1 torr (0.13 kPa). Microsomes were prepared from livers of male Wistar rats according to standard procedures [9]. Incubations contained 1 mg microsomal protein/ml and an NADPH-regenerating system [9].

Method A (conventional). Polyadenylic acid (mol.wt = 100,000; Serva, Heidelberg) was directly added to the microsomal incubations, at a concentration of 2 mg/ml. After 45 min of incubation at 37° the incubation vessels [9] were removed, chilled on ice, and the contents were transferred to ultracentrifugation tubes [6]. After centrifuging at 100,000 *g* for 1 hr, the supernatants containing the soluble components were dialyzed in dialysis bags at 4° for 36 hr against 0.1 M ammonium acetate buffer, pH 6.5. After dialysis, an aliquot was taken for determination of the remaining concentration on polyadenylic acid by spectrophotometry at 260 nm [6]. Subsequently, the whole was lyophilized and subjected to enzymic hydrolysis and chromatography.