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IN VITRO METABOLISM OF ZERANOL : EVALUATION OF COVALENT BINDING TO MICROSOMAL PROTEIN

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Résumé

MÉTABOLISME *IN VITRO* DU ZÉRANOL: ÉVALUATION DE LA LIAISON COVALENTE AUX PROTÉINES MICROSOMIALES. — Grâce à l'utilisation d'un modèle *in vitro*, à savoir les microsomes hépatiques bovins, les auteurs ont étudié l'oxydation du zéranol en zéaralanone ainsi que la formation de métabolites réactionnels et leurs liaisons aux protéines. Ces phénomènes étaient dus à l'intervention des monoxygénases hépatiques. Le niveau de la liaison covalente était très bas. Aucun effet de détoxification du glutathion et des glutathion-S-transférases n'a été mis en évidence.

The metabolism of zeranol has been studied in several species. An *in vitro* study by Ingerowski and Stan (1979) has shown that zeranol is oxidised to zearalanone by NADPH "nicotinamide adenine dinucleotide phosphate, reduced form" dependent monoxygenases in liver.

This metabolite has been identified as the main metabolite in all species studied. It is excreted with the parent drug in bile and in urine as glucuronide and (or) sulphate conjugates (Rico, 1983; Migdalof *et al.*, 1983) (fig. 1).

Covalent binding of zeranol with nucleic acid has been achieved (Barraud *et al.*, 1983). The covalent binding index (CBI) calculated in the rat was lower than the values obtained for trenbolone acetate and 17 β -estradiol.

For another anabolic drug, Ryan and Hoffmann (1978) have shown that covalent binding of trenbolone to protein materials occurs *in vivo*. This observation could explain the existence of bound residues in bovine liver and muscle.

In the case of trenbolone, hepatic monoxygenases produce reactive metabolite and this intermediate can be detoxified by glutathione conjugation (Evrard *et al.*, 1983).

In contrast, for zeranol there is no information about the production of reactive metabolites and the occurrence of bound residues (Rico, personal communication).

The present work deals with the evaluation of reactive metabolite production and of possible covalent binding of zeranol metabolite(s) to proteins using isolated bovine liver microsomes.

Materials and Methods

Chemicals

11,12 ³H zeranol (50 Ci/mmole), unlabelled zeranol and zearalanone were kindly provided by Dr Jouquey from Roussel Uclaf (Paris, France). Tritiated zeranol was mixed with unlabelled zeranol in methanol to obtain a specific activity of 5 Ci/mole.

NADP (nicotinamide adenine dinucleotide phosphate), glucose 6-phosphate, glucose 6-phosphate dehydrogenase and reduced glutathione were purchased from Boehringer (Mannheim).

Glutathione-S-transferases from bovine liver was from Sigma chemicals (USA).

All others chemicals were analytical grade reagents.

Preparation of bovine liver microsomes

All steps were carried out at 4 °C. Fresh bovine liver collected at the slaughter house was immediately chilled in cold, isotonic potassium chloride solution. Homogenization (Dounce homogenizer) of liver fragments was performed at 9000 *g* for 20 minutes (Beckmann J2-21 centrifuge; JA-20 rotor). Microsomes were isolated by centrifugation of the supernatant at 105000 *g* for 60 minutes using a Beckmann ultracentrifuge (rotor 50).

The microsomal pellet was suspended in 10 volumes of KCl solution, centrifuged again under the same conditions and finally suspended in 0.1 M phosphate buffer, pH 7.4 at a concentration of 4 mg protein per ml.

Protein content was determined by the method of Bradford (1976).

Incubation conditions

A homogenous solution of the substrate was prepared before use as follows: to a solution of zeranol (200 nmoles) in methanol (0.1 ml) was added 1 ml of a mixture of 0.1 M phosphate buffer, pH 7.4, and methanol containing 0.1 % Tween 80 (5 : 1). The mixture was heated at 50 °C and agitated vigorously.

The incubation mixture contained the microsomal suspension (2 ml), substrate solution (1 ml), NADP (1 mM), glucose 6-phosphate (5 mM), nicotinamide and magnesium chloride (5 mM each), glucose 6-phosphate dehydrogenase (1 IU) and 0.1 M phosphate buffer, pH 7.4 to a final volume of 10 ml.

Incubations were carried out at 37 °C for 20 minutes.

The reaction was stopped by addition of 3 volumes methanol followed by centrifugation at 10000 *g* for 30 minutes. Methanol-water supernatant fractions were collected and stored for further metabolite analysis. The pellets were used for evaluation of covalent binding.

A fraction of inactivated microsomes used for control was prepared in a boiling water bath for 10 minutes.

Some incubations were performed without cofactor (NADPH-generating system) and in the presence of glutathione (5 mM) or glutathione-S-transferase (1 mg).

Evaluation of covalent binding

Pellets were exhaustively washed with a mixture of methanol-ethylacetate (3 : 1) (Pohl and Branchflower, 1981).

Unbound radioactivity was removed after centrifugation at 10000 *g* for 30 minutes. After the last centrifugation, the pellet was dissolved in 1 ml Soluene-350 and 10 ml of Dimilume-30 (Packard, USA) was added.

Radioactivity was determined in a Beckmann LS-1800 counter.

Characterization of zeranol metabolite by Thin Layer Chromatography (TLC)

The methanol-water supernatant was extracted twice with chloroform and evaporated to dryness under vacuum. The dry residue was taken in methanol (0.5 ml).

An aliquot (0.025 ml) was layered onto a silica gel 60 TLC plate (Merck, Darmstadt) and eluted with chloroform-diethylether (35 : 15). Unlabelled zeranol and zearalanone were used as standards.

The chromatogram was stained by spraying a mixture of ethanol-sulphuric acid (95 : 5). The plate was heated at 110 °C for 10 minutes and spots were visualized with an UV-lamp (366 nm). The silica gel areas corresponding to visible spots were collected from the plate. They were extracted by addition of methanol (1 ml) and shook, picofluor 50 (5 ml) was added and the radioactivity was determined.

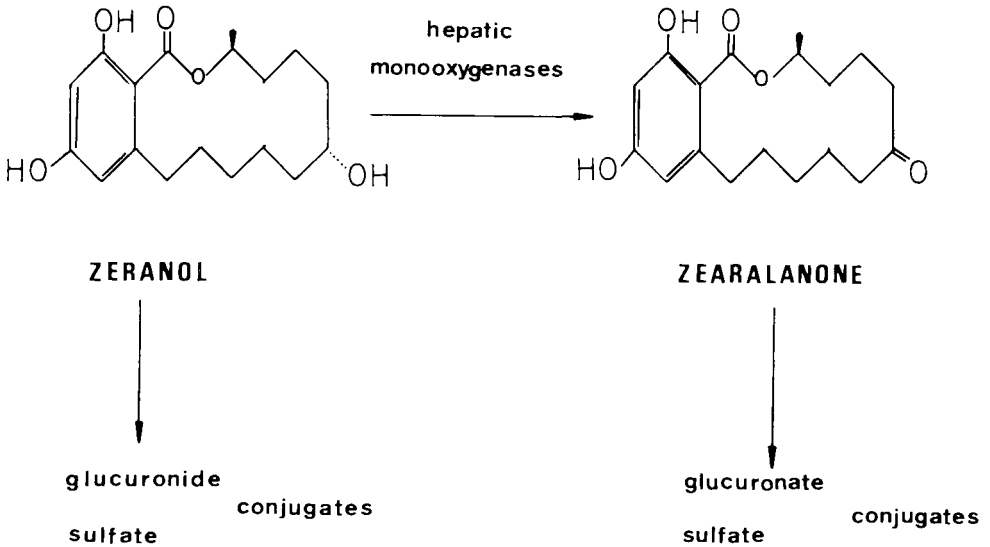


Fig. 1. — Metabolic pathway of zeranol.

Table 1. — Covalent binding of zeranol metabolite to protein in various conditions.

Experiment	Cofactor ^a	GSH ^b	GSH-S-T ^c	Covalent binding ^d
1	+	—	—	12.1 ± 0.26 (5)
2	—	—	—	11.9 ± 0.18 (5)
3	+	—	—	15.8 ± 0.39 (5)
4	+	+	—	15.4 ± 0.31 (5)
5	+	—	+	15.2 ± 0.29 (5)

a: NADPH-generating system

b: reduced glutathione

c: glutathione-S-transferases

d: values are the mean ± S.D. and are expressed in pM of zeranol equivalent per mg of microsomal protein.

Results

Covalent binding assays

The results of these experiments are given in table 1. Experiment 1 was a control, it represents the non-specific binding obtained with denatured microsomes. Covalent binding in experiment 2 was not significantly different from experiment 1. Experiment 3 measured the total covalent binding; results were significantly different from those of experiments 1 and 2 ($P < 0.05$).

Values obtained in experiments 4 and 5 were not significantly different from total binding value.

Characterization of zeranol metabolite by Thin Layer Chromatography (TLC)

TLC analysis of methanol-water fractions from experiments 1 and 2 does not show any oxidation in zearalanone.

In the last three experiments (3, 4, 5), each sample showed four spots, two of them being radioactive. The *rf* and the colour fluorescence (yellow-green) of these two spots were identical to that recorded for authentic samples of zeranol (*rf* = 0.091) and zearalanone (*rf* = 0.32). The rate of zeranol biotransformation into zearalanone was calculated using radioactivity data. It was found to be equal to 1.5 nM per minute per mg of microsomal proteins.

Zeranol/zearalanone radioactivity ratio was estimated to 0.089 and did not decrease with longer incubation time.

Discussion

Assessment of the activity of NADPH-dependent monooxygenases was provided by the observed high rate of zeranol oxidation to zearalanone.

The observed covalent binding of zeranol metabolite to microsomal proteins was very low (i.e. 3.7 pM per mg of microsomal proteins) and could be interpreted as a very low metabolic activation of zeranol.

The NADPH-dependent monooxygenases are involved in this electrophilic intermediate production since the absence of cofactor decrease bound radioactivity to the level of non-specific binding.

A role of glutathione and glutathione-S-transferases in the detoxication of electrophilic metabolites has been suggested (Wolkoff *et al.*, 1980; Orrenius and Jones, 1978).

In the present work, we have no evidence of such a mechanism, as reactive metabolite is produced only at a very low rate.

Using the same experimental conditions, covalent binding of trenbolone acetate is about 250 times higher (Evrard *et al.*, 1983). Therefore, the values obtained for zeranol might be considered as negligible. It is thus most probable that the level of bound residues following chronic treatment (i.e. implantation) in living animals will remain low.

Moreover, as these kind of residues have lost their reactivity (Burgat-Sacaze *et al.*, 1981) and exhibit a low bioavailability (Ross, 1981) a toxic effect of "zeranol-bound" residues for the human consumer of meat is most improbable.

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Summary

Using an *in vitro* system (i.e. bovine liver microsomes), the authors have studied the metabolism of zeranol. The oxidation of zeranol into zearalanone by NADPH-dependent monooxygenases has been measured. The rate of metabolic activation and the level of covalent binding to microsomal proteins of zeranol metabolites have been evaluated. They were found to be very low. A possible detoxication effect of glutathione and glutathione-S-transferases was undetectable.

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