Metabolism and Disposition of [^{3}H]Zeranol Implanted in the Pig

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The metabolic fate and distribution of [^{3}H]zeranol has been studied in the pig following implantation in association with trenbolone acetate. A steady state was reached after 7 days. Plasma tritiated water was less than 1%. The metabolic balance established on the 13th day shows a very predominant urinary excretion (62.7–80%). A considerable biliary excretion presumes the existence of an intense enterohepatic circulation. Free zeranol, zearalanone, and taleranol as well as the corresponding glucurono- and sulfoconjugates have been identified in the urine, bile, and feces, which confirms our previous in vitro studies. Quantitation indicates glucuronoconjugates are the major metabolites and zearalanone is the major aglycon. Liver is the target tissue, and most of the residual radioactivity corresponds to similar quantities of zeranol and its two metabolites under conjugated form.

Zeranol [7α-zearalanol or [3*S*-(3*R**,7*S**)]-3,4,5,6,7,8,9,-10,11,12-decahydro-7,14,16-trihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1-one (Figure 1)] is a nonsteroidal substance used alone (Ralgro) or associated with trenbolone acetate (Forplix) as a growth promoter in cattle and sheep. Application to pig has been envisaged too (Denzer et al., 1986). The disposition and metabolism of [11,12-^{3}H]zeranol has been studied in the rat, rabbit, dog, rhesus monkey, and human volunteers, after oral administration (Migdalof et al., 1983). This compound is absorbed to a large extent, but considerable biliary excretion has been measured in the rat. Important interspecies variations of the distribution between urine and feces have been observed, i.e., major urinary excretion in the rabbit and man but predominant fecal elimination in the rat, dog, and monkey. The main metabolite identified in the urine, bile, and feces was zearalanone resulting from the oxidation of zeranol at C-7. β-Zearalanol or taleranol was identified in the rabbit urine, and a monohydroxylated metabolite of zeranol was isolated from the human urine but its identification has not been completed. Moreover, zeranol and zearalanone were conjugated to various extents as glucuronides and/or sulfates.

Recently we have shown that after [^{3}H]zeranol implant administration to the pig, zeranol, taleranol, and zearalanone appeared in the plasma as free and conjugated metabolites (Bories and Fernandez-Suarez, 1989). Moreover, pig hepatocyte subfractions incubated with [^{3}H]-zeranol, taleranol, and zearalanone were shown to produce monosulfo- and monoglucuronoconjugates (Bories et al., 1991).

The present study examines the metabolic fate and distribution of [^{3}H]zeranol administered to the pig as an implant in association with trenbolone acetate, on the basis of excreta, bile, and tissue analysis.

MATERIALS AND METHODS

Chemicals and Equipment. Zeranol and zearalanone were supplied by Roussel-Uclaf (Romainville, France). Taleranol was prepared according to the method of our previous work (Bories et al., 1991). Glucuronidase bacterial type II and helicase (type H-2 from *Helix pomatia*) were obtained from Sigma (St. Louis, MO). Acetonitrile and dichloromethane (HPLC grade) were purchased from E. Merck (Darmstadt, FRG), and bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Europe (Oud-Beijerland, The Netherlands). 1-Heptanesulfonic acid (PIC B7) was from Waters (Milford, MA).

The HPLC system used consisted of a Waters pump system with a Spherisorb ODS-1 C_{18} (5 μm) column (250 mm × 0.4 mm i.d.), and the eluate was collected with a Gilson Model 202 fraction collector. A Minaxi 4430 liquid scintillation spectrometer (Packard, Downers Grove, IL) was used for radioactivity measurements. The fluor mixture used was ACS II from Amersham (Amersham, U.K.). A Hewlett-Packard (Palo Alto, CA) Model 5992 B gas chromatograph–mass spectrometer in the electron impact (EI) mode was used for structural confirmation.

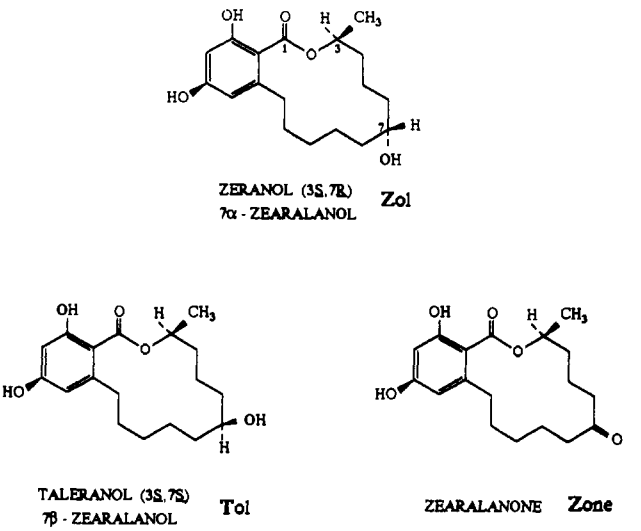

Figure 1. Structures of zeranol and related metabolites.

Labeled Compounds. Labeled implants containing 20 mg of trenbolone acetate plus 36 mg of zeranol including 2.22 GBq of [11,12-^{3}H]zeranol (25.9 GBq/mmol) were prepared by Roussel-Uclaf.

Zeranol, taleranol, and zearalanone [^{14}C]monoglucuronates were prepared by incubation of the standard steroid-like compounds with [^{14}C]UDP-glucuronic acid in the presence of pig liver microsomal subfractions (Bories et al., 1991). Zeranol, taleranol, and zearalanone [^{35}S]monosulfates were prepared by incubation of the same compounds with [^{35}S]sodium sulfate in the presence of pig liver cytosolic subfractions according to the method of the same work. Aliquots of the incubates were used directly for the calibration run of the metabolic profiles.

Animal Treatment. One male and one female large White × Landrace pig about 55 kg (live weight) were acclimated to metabolic cages for a week before they received the labeled subcutaneous ear implant. Blood sampling was performed once a day for 13 days following the implantation using a catheter placed in the carotid artery, and the plasma was prepared immediately by centrifugation. Urine was collected quantitatively for 24 h on day 13 over 500 mL of methanol and then kept at −30 °C. Feces were collected quantitatively during the same

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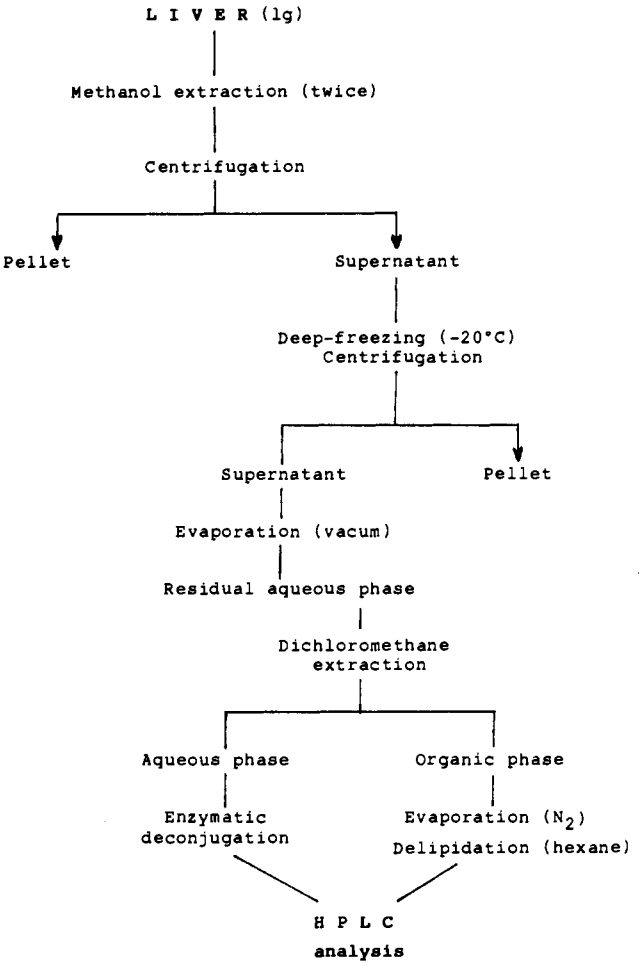

Figure 2. Extraction procedure for liver [3H]zeranol residues.

period and kept at −30 °C. The animals were slaughtered on day 14, and samples of bile were collected and kept at −30 °C.

Analytical Procedures. *Radioactivity Measurements.* Plasma, bile, and urine aliquots were counted directly by liquid scintillation counting. Feces were homogenized, lyophilized, and then ground using a coffee grinder. Aliquots (250 mg) were combusted in an oxidizer (Oxymat, Intertechnique), and the resulting tritiated water was measured by liquid scintillation counting. Tritiated water content of the urine was evaluated from radioactivity measurements before and after lyophilization.

Extraction and Cleanup Procedure for Bile, Feces, and Tissue (Liver). One milliliter of bile was diluted with 10 mL of water and adjusted to pH 1 using 5% hydrochloric acid. Liquid–liquid extraction was performed using 15 mL of butanol. The butanolic phase was separated and evaporated to dryness under vacuum. The residue was dissolved in the appropriate HPLC mobile phase (see below).

One gram of lyophilized feces was vortexed with 10 mL of a water/methanol (60:40) mixture. After centrifugation at 4000*g* for 10 min, the supernatant was recovered and the pellet was extracted another time with an additional 10 mL of the solvent mixture. The two supernatants were gathered and evaporated to dryness under vacuum. The residue was dissolved in the appropriate HPLC mobile phase (see below).

Liver samples were handled according to the method shown in Figure 2. It can be summarized as a methanol extraction followed by a partition between low-polarity compounds and conjugates.

Enzymatic Deconjugation. To 1 mL of urine, bile, or liver extract final aqueous phase, either 1 mL of phosphate buffer (pH 6.8) and 1 mL of bacterial glucuronidase (about 200 UI) or 1 mL of phosphate buffer (pH 5.5) and 1 mL of helicase (1000 UI) were added. After overnight incubation at 37 °C, the free metabolites were extracted using dichloromethane and then analyzed by radio-HPLC.

HPLC Analysis. Three solvent mixtures were used to separate free and conjugated metabolites. Solvent A consisted of 0.2% acetic acid–methanol–PIC B7 (70:30:0.4). Solvent B consisted of 0.2% acetic acid–methanol–PIC B7 (60:40:0.4). Solvent C consisted of 0.2% acetic acid–methanol–acetonitrile (50:20:30).

The following gradient program was used: initial A (100%); 4 min A (100%) to B (100%); 12 min B (100%); 0.2-min step B (100%) to C (100%); 30 min C (100%).

Flow rate was 1 mL/min for all analyses. Fractions were collected every 20 s in 3-mL polyethylene tubes, and then 2 mL of the fluor mixture were added.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis. HPLC peaks of the free metabolites were collected, the mobile phase was evaporated to dryness under a flow of nitrogen, and the residue was derivatized using BSTFA at room temperature. The mixture was injected through a Ross injector into the GC–MS system equipped with a 12.5-m OV-1 capillary column and submitted to a 150–250 °C (5 °C/min) temperature-programmed run.

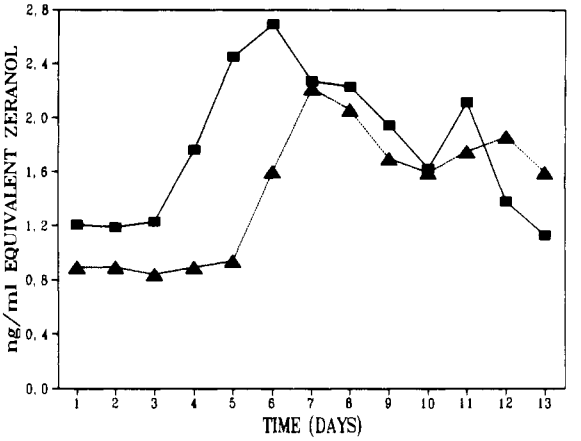

Figure 3. Plasma radioactivity kinetics for pigs implanted with [3H]zeranol. (■) Female pig; (▲) male pig.

RESULTS

Plasma Kinetics. The kinetics of the plasma radioactivity following [3H]zeranol implant administration is reported in Figure 3. It shows the very classical two-step curve, i.e., increase in blood concentration followed by a depletion which corresponds to the release of the implant. It is remarkable that lag times of 5 (male) and 3 days (female) are observed before this phenomenon occurs. This may be explained by the fact that the implant is a foreign body placed in subcutaneous areas of the ear with limited blood irrigation. Therefore, it takes some time for a new tissue to develop and to ensure a narrow contact with the implant that will favor the diffusion of the anabolic. A metabolic equilibrium was reached after 7 days. The maximum plasmatic concentrations were about 2.68 (male) and 2.22 ng/mL (female) expressed as zeranol.

Tritiated water content was evaluated from the loss of radioactivity of plasma samples submitted to lyophilization. It represented less than 1% of the total plasma radioactivity, which confirms the chemical and metabolic stability of the tritium-labeled positions of the molecule already reported (Migdalof et al., 1983).

Metabolic Balance. Total urinary and fecal excretion was measured on day 13 following implantation, i.e., when constant diffusion of the implant and metabolic equilibrium was established. The results are presented in Table I. The total radioactivity excreted was different for the two animals; however, the ratios of total blood content vs total excretion gave similar values (9.9×10^{-3} and 11.8×10^{-3}, respectively), meaning that emptying of the implant was more advanced in the female. If one hypothesizes that no significant accumulation of zeranol or metabolites occurs in the animal during this initial 13-day postimplantation phase, it can be assumed that the radioactivity

Table I. Metabolic Balance of [^{3}H]Zeranol in Male and Female Pigs (13 Days Postimplantation)

	total radioactivity expressed as zeranol equivalent, mg	
	male	female
urine	0.802	0.355
feces	0.215	0.211
total	1.017	0.566

excreted corresponds to the radioactivity released from the implant. For that particular day, it represents 2.83% (male) and 1.57% (female) of the initial implant contents, respectively. The distribution of the radioactivity between the urine and feces indicates a very predominant urinary excretion (male, 80%; female, 62.7%).

Total radioactivity in bile expressed as zeranol was equivalent to 350 ng/mL for both animals. The extrapolation of this value to a 3-L daily excretion figure for 60-kg (live weight) pigs shows that about 1 mg of zeranol equivalent could be excreted in this way. Such a quantity is by far higher than that excreted through either the urine or the feces, which presumes the existence of an intense enterohepatic circulation.

Identification of Urinary, Fecal, and Biliary Metabolites. The radio-HPLC profiles obtained from male and female pig urines are shown in Figure 4. The calibration was achieved by using standard taleranol, zeranol, and zearalanone and each of the subcellular fraction incubates prepared to produce the corresponding monoglucurono- and monosulfoconjugates. The step by step elution conditions used allowed the nearly complete recovery (94.4%) of the total radioactivity of the urine samples that were directly injected on the column. Three distinct areas appeared on the profiles.

Area I shows three peaks whose retention times correspond to those of standard zearalanone, zeranol, and taleranol. Confirmation of the identity of these metabolites (collected by HPLC) was obtained from the GC–MS analysis of the silylated derivatives. Identical spectra were obtained for the supposed zeranol and taleranol with [M′] = [M + 3TMS] = 538, [M′ − 15] = 523, [M′ − OTMS − 15] = 433 and [M − 15] = 307, which agrees with our previous work (Bories and Fernandez-Suarez, 1989). The third metabolite exhibited m/z = 464, 449, and 307, which corresponds to trimethylsilylated zearalanone (Covey et al., 1988; Bories et al., 1989). A peak with lower retention time was observed, which corresponds to an unknown and more polar compound.

Area II exhibits three major peaks whose retention times correspond to taleranol, zeranol, and zearalanone glucuronoconjugates. Following enzymatic deconjungation using β-glucuronidase, all of the peaks from area II disappeared, giving rise to the three peaks identified in area I, which confirms they correspond to glucuronoconjugates.

Area III shows three peaks whose retention times correspond to zearalanone, zeranol, and taleranol sulfoconjugates. In male urines, a minor peak with lower retention time corresponds to very polar unidentified compounds that are practically not retained on the column.

From a qualitative point of view, both the male and female profiles are similar. The distributions between zeranol, zearalanone, and taleranol in terms of total radioactivity as well as of free or conjugated metabolites are relatively similar (Table II). Glucuronides are by far the major conjugates, but significant quantities of sulfoconjugates have been measured. The unknown metabolites are below 5% in both the male and female.

A complete recovery of the fecal radioactivity was obtained using the extraction procedure described. The analysis of the fecal extracts of the male and female pigs indicates that most of the radioactivity (94.4% and 94.1%, respectively) is associated with free zeranol and metabolites. Only a small fraction (3.1% and 4%) behaves as conjugates. However, due to the very limited quantity of residual radioactivity available at the end of the necessary cleanup steps, it was not possible to further investigate the nature of these supposed conjugates. The radio-HPLC profile of the feces extract of the male (Figure 5) shows that zeranol is the major compound (54.3%), while zeralanone and taleranol represent 38.0% and 2.1%, respectively. In the female pig, zearalanone is the preeminent metabolite (63.2%), while zeranol represents 26.8% and taleranol 4.1%.

Bile extraction using the procedure described allowed 90.2% recovery of the radioactivity. Bile extract analysis showed that free zeranol or metabolites were absent. The radio-HPLC profile obtained from the male pig (Figure 6) indicates that zearalanone monoglucuronoconjugate is the main metabolite (79.4% of the total radioactivity of the extract), while zeranol glucuronide (9.8%) and taleranol glucuronide (3.1%) are the main other radioactive compounds. The sulfoconjugates of taleranol and zearalanone are present in very small quantities (4%). Unknown compounds are present in the bile at a limited extent (4.5%). A similar distribution is observed in the bile of the female pig, i.e., 64.9%, 22.9%, and 4.5%, respectively, for the three compounds but with an increased zeranol vs zearalanone glucuronoconjugate ratio.

Identification of Tissue Liver Metabolites. The total radioactivity measured in the tissues of the male and female pigs slaughtered 14 days following implantation, expressed as zeranol, indicates that kidney (8.8 and 12.8 ng/g, respectively) and liver (7.0 and 5.0 ng/g, respectively) are the target tissues. The corresponding figures for the other tissues are as follows: muscle (0.6 and 0.4 ng/g); fat (1.2 and 1.7 ng/g).

About 90% of the radioactivity of the liver was extractable. Free zeranol plus metabolites represented only 8.7% and 2.9% of the total radioactivity in the male and female, respectively, and taleranol was the major free metabolite. Following helicase treatment, 92% and 93.7%, respectively, of the extractable radioactivity was recovered as zeranol, zearalanone, and taleranol (Figure 7). The repartition between these metabolites in their free or conjugated form is given in Table III. It shows very similar distributions in both animals, and zeranol, zearalanone, and taleranol are present in equivalent proportions.

DISCUSSION

No previous data on blood level, tissue distribution, and excretion of zeranol administered to the pig have been reported. The present study has confirmed and extended the results obtained by Migdalof et al. (1983) in other species, even if the mode of administration, i.e., implantation instead of oral dosage and simultaneous application with trenbolone acetate, was different. The balance study indicates that urine represents the major excretion pathway as in the rabbit or in man. The massive biliary excretion observed in the rat, especially following intracardiac administration, is observed in the pig also. Moreover, the fact that the bile and feces contain nearly exclusively conjugates for the former and free metabolites for the latter leads to the conclusion that an intense enterohepatic circulation occurs. It must be emphasized that both the male and female animals behave similarly.

The present study has confirmed and extended the preliminary results obtained from plasma metabolic profiling (Bories and Fernandez-Suarez, 1989) and in vitro

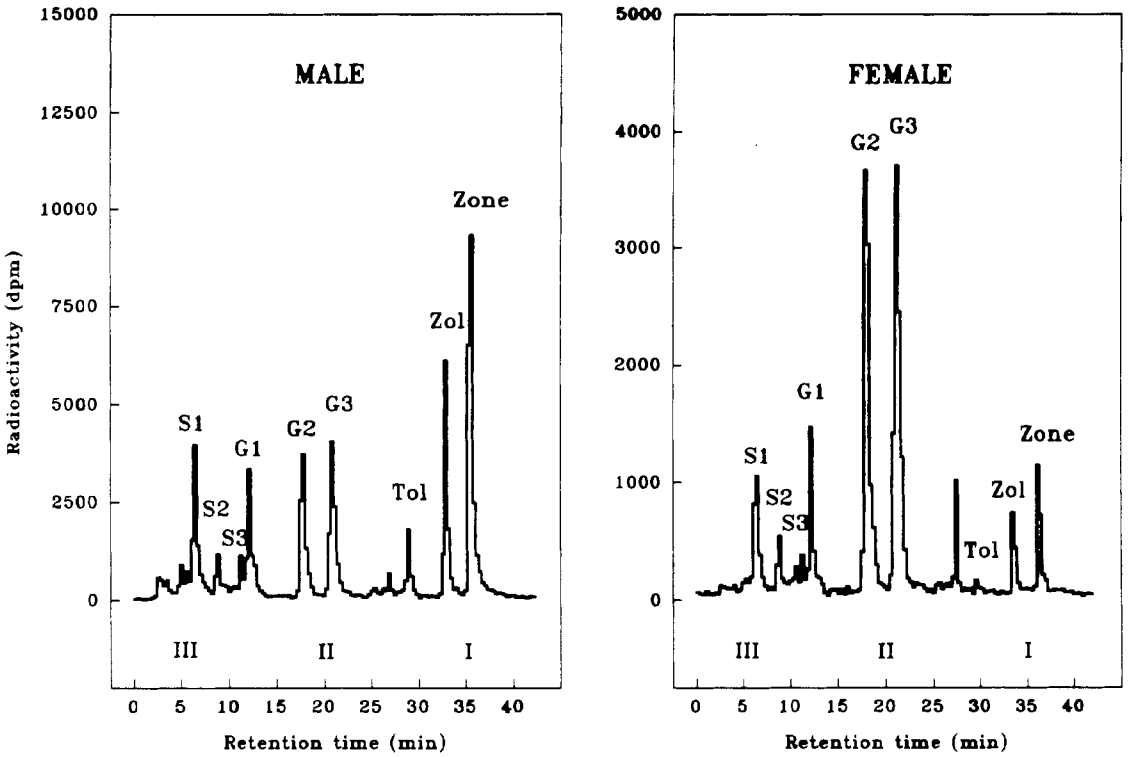

Figure 4. Urinary metabolic profiles of male and female pigs implanted with [^{3}H]zeranol. S1, S2, and S3 are the respective sulfates of Tol, Zol, and Zone. G1, G2, and G3 are the respective glucuroconjugates of Tol, Zol, and Zone.

Table II. Relative Amounts of [^{3}H]Zeranol and Principal Metabolites in Pig Urine at 13 Days Postimplantation

		zeranol	zearalanone	taleranol
free metabolites, %	M	27.8	64.8	7.4
	F	35.5	58.8	5.7
conjugated metabolites, %	M	34.9	43.3	21.8
	F	41.5	49.0	9.5
total metabolites, %	M	32.6	48.7	17.7
	F	40.7	50.3	9.0

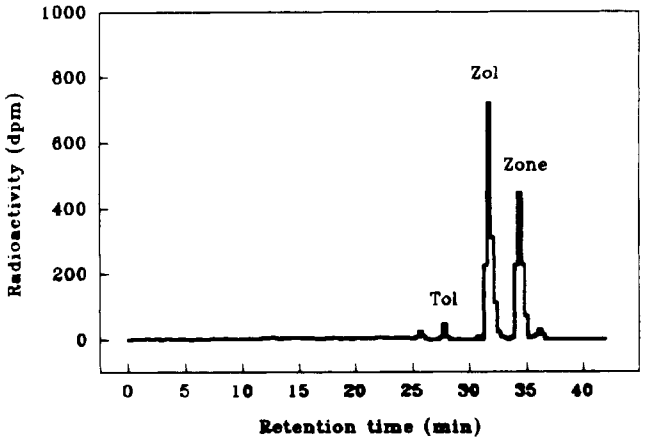

Figure 5. Fecal metabolic profile of male pig implanted with [^{3}H]zeranol.

study with pig hepatocyte subcellular fractions (Bories et al., 1991). Zeranol is extensively metabolized in vivo to free and conjugated metabolites. The main metabolic pathways previously described or hypothesized for different species (Migdalof et al., 1983) can be extended to the pig. Oxidation at C-7 leads to the corresponding ketone, zearalanone. The reverse reaction occurs through aldoketoreductase action (Bachur, 1976). Reduction of zearalanone generates a mixture of the parent zeranol and its epimer taleranol. Whether the observed ratios depend on the biotransformation process only or result from both

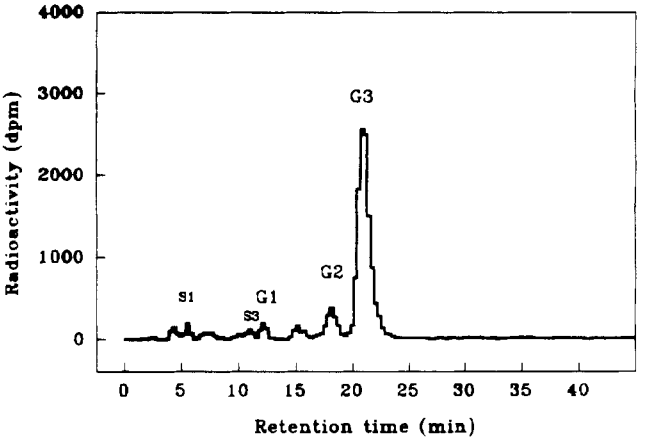

Figure 6. Biliary metabolic profile of male pig implanted with [^{3}H]zeranol. S1, S2, and S3 are the respective sulfates of Tol, Zol, and Zone. G1, G2, and G3 are the respective glucuroconjugates of Tol, Zol, and Zone.

enzymatic reaction and thermodynamic equilibrium of the diastereoisomers remains to be elucidated. It must be noted that Migdalof et al. (1983) have measured appreciable amounts of taleranol in rabbit urine only. Studies performed in the pig with the very structurally similar mycotoxin zearalenone administered orally have shown that the α and β epimers were excreted in the urine (Mirocha et al., 1981).

Another issue of this study is the fact that, as already demonstrated for the other species (Migdalof et al. 1983), conjugation of zeranol and metabolites occurs to a large extent in the pig. The difference observed between the male and female concerning the proportion of free and conjugated metabolites excreted in the urine should be confirmed using a greater number of animals. As a matter of fact, the multispecies metabolic study of the former authors was carried out using female animals only. Moreover, despite the fact that normal care was taken to avoid bacterial development during urine collection and storage, one may admit that bacterial deconjugation may

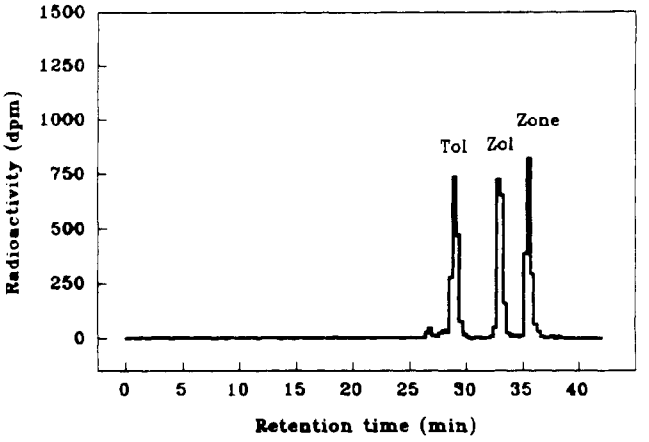

Figure 7. Liver residue profile of male pig implanted with [^{3}H]-zeranol (13 days postimplantation, helicase deconjugation).

Table III. Relative Amounts of [^{3}H]Zeranol and Principal Metabolites in Pig Liver at 14 Days Postimplantation

		zeranol	zearalanone	taleranol
free metabolites, %	M	2.5	2.1	4.1
	F	1.0	0.8	1.1
conjugated metabolites, %	M	32.1	32.2	27.0
	F	38.5	34.1	24.5

occur in certain circumstances in the urinary tract. Although glucuronoconjugation appears to be the major metabolic pathway, the isolation and identification of sulfoconjugate from the urine confirm our preliminary in vitro study (Bories et al., 1991) and the Migdalof et al. (1983) hypothesis. It must be noted that no sulfate excretion was observed in the piglet that received zearalenone orally when the same analytical methodology which proved to be efficient in identifying these compounds in cow urine (Mirocha et al., 1981) was used.

The nonaccounted for radioactivity was very limited in both the male and female urines and nonsignificant in the bile, feces, or tissues. Much larger amounts of unknown compounds were found in man and rabbit urines by Migdalof et al. (1983). The authors hypothesized these polar compounds could arise from the hydroxylation of zeranol or hydrolysis of the lactone of zearalanone to provide the ring-opened keto acid.

The investigation on the distribution of the radioactivity in the target tissue, i.e., the liver, emphasizes the fact that most of the residues are extractable and that free zeranol and metabolites represent only a very minor fraction. However, it must be noted that this study concerns pig tissues sampled in the initial phase of zeranol treatment and cannot anticipate the residual status at the end of the efficacious period of the implant, i.e., after 1 or 2 months. Another major consideration is the fact that zeranol is associated with trenbolone acetate in the implant. Whether this steroid-like substance that is metabolized through proximate metabolic pathways, namely reduction to the corresponding diastereoisomeric alcohols and glucuronoconjugation (Pottier and Cousty, 1981), interferes with the biotransformations or blood transportation of zeranol as a competitive substrate remains to be investigated. If it was the case, the quantitative incidence on the residual status of zeranol could be of major importance when compared to the application of the compound alone.

The helicase treatment proved to be very efficient for the cleavage of the conjugates, which makes the use of this enzyme mixture or the association of β-glucuronidase and sulfatase (Verbiese-Genard and Hanocq, 1986; Kim et al., 1986) compelling for the analytical procedures designed to determine zeranol and related residues in tissues. It is remarkable that taleranol was present in about the same amount as zeranol. When one considers that the estrogenic potency of taleranol is only half that of zeranol but slightly higher than that of zearalanone (Everett et al., 1987), taleranol residues must be taken into account in the calculation of the no-hormone effect level, which implies the necessity for the development of analytical methods able to measure zeranol, zearalanone, and taleranol separately.

In summary, the present study performed in an animal species not investigated until now clearly shows that the pig exhibits the same metabolic capabilities described for the other species. Moreover, it confirms our previous in vitro studies showing for the first time that zeranol and its metabolites zearalanone and taleranol were conjugated to a large extent as monoglucurono- and monosulfoconjugates. Another feature is the determination of the nature of the liver residues, which underlies the development of appropriate analytical procedures.

ACKNOWLEDGMENT

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