Journal of Chromatography, 489 (1989) 191–197
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4608

PROFILING OF FREE AND CONJUGATED [^{3}H]ZERANOL METABOLITES IN PIG PLASMA

GEORGES BORIES* and ADRIANA FERNANDEZ SUAREZ

INRA, Laboratoire des Xénobiotiques, 180 ch. de Tournefeuille, B.P. 3, 31931 Toulouse Cedex (France)

SUMMARY

Extraction and high-performance liquid chromatographic (HPLC) procedures are described that permit the complete analysis of free and conjugated zeranol metabolites in plasma from pigs implanted with [^{3}H]zeranol. Free metabolites (9.0%) are extracted and then analysed by radio-HPLC on a reversed phase C_{18} column. They are distributed between three compounds that have been identified by gas chromatography–mass spectrometry as taleranol, zeranol and zeralanone. Direct radio-HPLC of the pre-extracted, deproteinated and Sep-Pak C_{18}-purified plasma on a reversed-phase C_{18} column using tetrabutylammonium as an ion-pairing agent showed four main peaks: one corresponds to a weakly retained unidentified compound(s) (20%) and the other three were identified as the taleranol, zeranol and zeralanone glucuro conjugates. However, the total recovery is only about 25% owing to strong affinity of this polar material for the plasma proteins. Enzymatic deconjugation of the pre-extracted plasma followed by radio-HPLC analysis of the freed metabolites led to a good recovery of the radioactivity (81.8%) and allowed the quantitation of the different metabolites. These preliminary results indicate that zeranol is metabolized in the pig following pathways similar to those in other tested species.

INTRODUCTION

Zeranol [6-(6,10-dihydroxyundecyl)-β-resorcylic acid lactone] is a non-steroidal substance used as a growth promoter in food-producing animals. It is approved for use in the U.S.A. in beef cattle as a 36-mg dose for subcutaneous ear implantation and for lamb as a 12-mg dose, which increase the rate of weight gain and improve feed conversion. Association of zeranol with the androgenic compound trenbolone acetate is under investigation in both species, and application to the pig has been envisaged [1]. The metabolism, toxicology and methods for the detection of residues have been reviewed [2]. The disposition and metabolism of [^{3}H]zeranol has been studied in the rat, rabbit, dog, monkey and human volunteers following oral administration [3]. The major metabolite identified was

0378-4347/89/$03.50

zeralanone, resulting from 7-OH oxidation. The diastereoisomer 7β-zeranol (taleranol) was also identified in the rabbit. It has been shown that zeranol and its metabolites undergo extensive conjugation as glucuro and/or sulpho conjugates. Studies with cattle and sheep have not been published [2], except for a balance study and plasma pharmacokinetics in steers [4]. In vitro studies [5] have shown that bovine liver microsomes are able to oxidize zeranol to zeralanone.

The analytical methodology available for investigating the fate of zeranol in vivo includes thin-layer chromatography [6] and very sensitive radioimmunoassays exhibiting more or less extended specificity towards zeranol and zeralanone [7–9]. High-performance liquid chromatography (HPLC) has been used to determine zeranol and zeralanone in spiked meat samples simultaneously [10] and to separate zeranol, taleranol and zeralanone in cattle urine [11]. Zeranol conjugates have been analysed, but only following enzymatic cleavage.

Metabolic profiling of [^{3}H]zeranol in plasma involving a complete HPLC analysis of free and conjugated [^{3}H]zeranol metabolites is proposed for the investigation of the biotransformation of this substance when administered to the pig as an implant of 36 mg of zeranol plus 20 mg of trenbolone acetate.

EXPERIMENTAL

Chemicals and equipment

Zeranol and [11,12-^{3}H]zeranol (700 mCi/mmol) were supplied by Roussel-Uclaf (Romainville, France). Glucuronidase bacterial type II and helicase (type H-2 from *Helix pomatia*) were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and dichloromethane (HPLC grade) were purchased from Carlo Erba (Milan, Italy), methanol (HPLC grade) from Merck (Darmstadt, F.R.G.) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce Europe (Oud-Beijerland, The Netherlands). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

The HPLC system used consisted of a Waters pump system with a Spherisorb ODS-1 C_{18} (5-μm) column (250 mm $\times$ 0.4 mm I.D.), and the eluate was collected with a Gilson Model 202 fraction collector. A Tricarb 4000 liquid scintillation counter (Packard, Downers Grove, IL, U.S.A.) was used for radioactivity measurements. The fluor mixture used was ACS II aqueous counting scintillant from Amersham (Amersham, U.K.). A Hewlett-Packard (Avondale, PA, U.S.A.) Model 5992 B gas chromatograph–mass spectrometer in the electron-impact (EI) mode was used for structural confirmation.

Animal treatment

A female pig, 55 kg live weight, received a subcutaneous implant of a pellet containing 36 mg of zeranol plus 20 mg of trenbolone acetate in the ear, including 70 mCi of tritiated zeranol. Blood sampling was performed on the thirteenth day following the implantation using a catheter placed in the carotid artery. The plasma was prepared immediately by centrifugation and a 0.1-ml aliquot was taken for total radioactivity measurement.

Extraction procedure

The overall procedure can be divided into three major steps (Fig. 1): (a) extraction and reversed-phase HPLC analysis of the free metabolites; (b) deproteination of the residual plasma and direct ion-pair HPLC of the polar (conjugated)metabolites; (c) enzymatic hydrolysis of the plasma followed by solvent extraction of the freed metabolites and HPLC analysis as in (a).

Free metabolites. Plasma (1 ml) was extracted twice using 10 ml of dichloromethane. After shaking for 20 min followed by centrifugation for 10 min at 4000 *g*, the organic phase was recovered and evaporated to dryness under a flow of

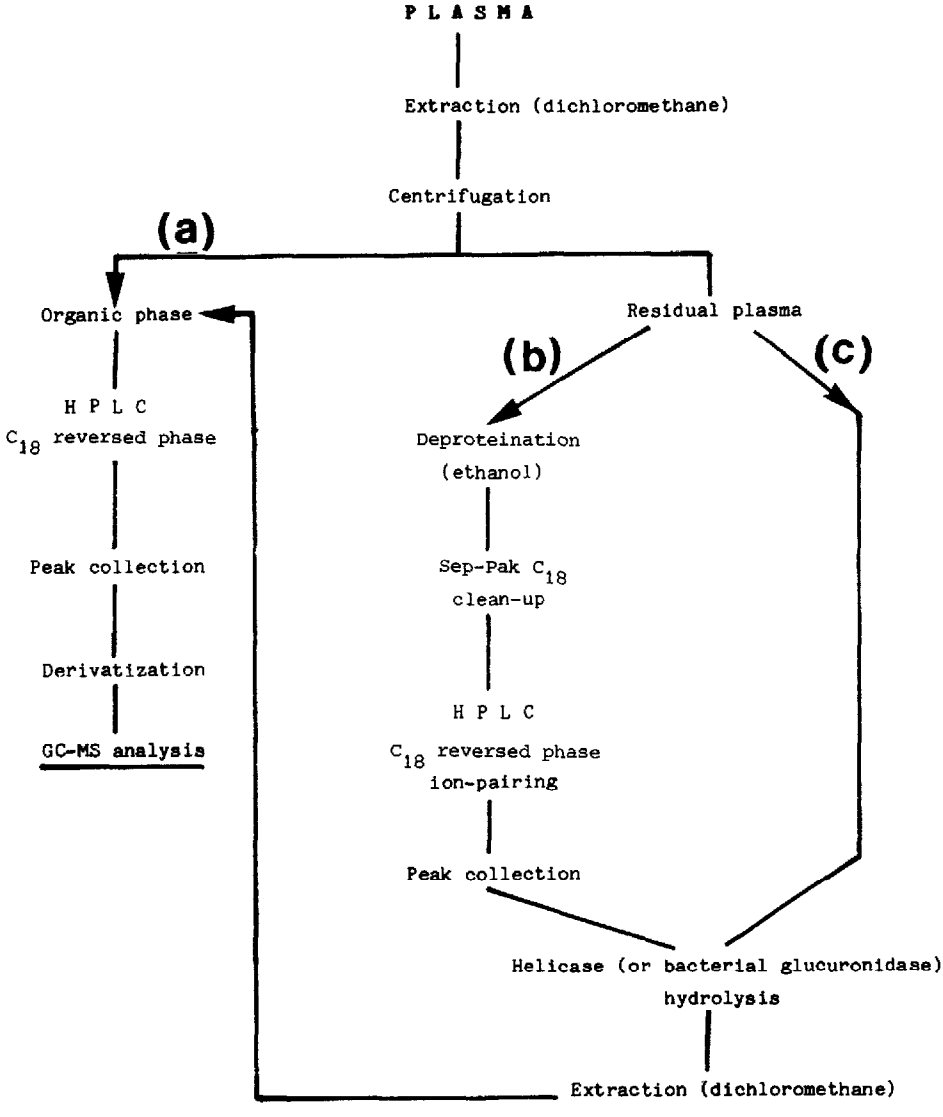

Fig. 1. Scheme of the analytical procedure for the isolation and identification of [^{3}H]zeranol metabolites in pig plasma.

nitrogen. The residue was dissolved in the HPLC mobile phase and submitted to chromatography, fraction collection and radioactivity counting.

Conjugated metabolites. Following extraction of free metabolites, 2 ml of residual plasma were deproteinated by adding 10 ml of ethanol. After shaking for 20 min, vortex mixing for 2×30 s and centrifugation for 10 min at 4000 *g*, the supernatant was recovered and the same operation was repeated after the addition of another 5 ml of ethanol. The deproteinated ethanolic plasma was evaporated to dryness under a flow of nitrogen. The residue was dissolved in 1 ml of phosphate buffer (pH 5.3) and passed through a Sep-Pak C_{18} disposable column preconditioned using water (6 ml), methanol (6 ml) and water (6 ml) successively. The radioactivity was eluted using 6 ml of methanol. The eluate was evaporated under vacuum and the residual water was eliminated using additional ethanol. The residue was dissolved into 100 μl of HPLC mobile phase and submitted to chromatography followed by fraction collection and radioactivity counting.

Enzymatic deconjugation. To 1 ml of plasma freed from the solvent-extractable metabolites was added either 1 ml of phosphate buffer (pH 6.8) and 1 ml of bacterial glucuronidase (about 200 U) or 1 ml of phosphate buffer (pH 5.5) and 1 ml of helicase (1000 U). After overnight incubation at 37 °C the free metabolites were extracted using dichloromethane and then analysed by radio-HPLC.

HPLC analysis

Free metabolites were separated on a reversed-phase column using 0.2% acetic acid–acetonitrile–methanol (50:30:20) as mobile phase at a flow-rate of 1.2 ml/min.

Conjugated metabolites were chromatographed on a reversed-phase column using tetrabutylammonium as ion-pairing agent. The mobile phase was aqueous tetrabutylammonium solution (2.22 g/l)–methanol (60:40) adjusted to pH 7.2 with phosphoric acid. The flow-rate was 1 ml/min. For both analyses 0.18-ml fractions were collected in 3-ml polyethylene tubes, then 2 ml of fluor mixture was added.

Gas chromatographic–mass spectrometric (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.

RESULTS AND DISCUSSION

On the thirteenth day following the implantation of the zeranol–trenbolone acetate pellet in the female pig, the total plasma radioactivity expressed as zeranol corresponded to 7.9 ng/ml. Solvent-extractable free metabolites represented 9.0% of the total radioactivity of the plasma. The radio-HPLC metabolic profile obtained is shown in Fig. 2. The chromatographic separation is comparable to that obtained using a similar reversed-phase column but a different mobile phase

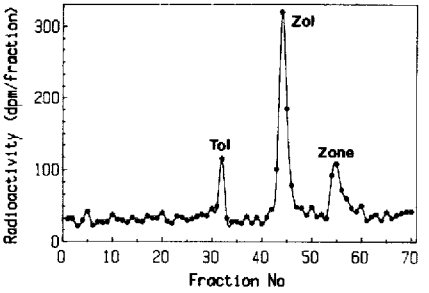

Fig. 2. HPLC metabolic profile of free metabolites of [^{3}H]zeranol in pig plasma. Chromatographic conditions: Spherisorb ODS-1 C_{18} (5 μm) column (250 mm × 0.4 mm I.D.); mobile phase, 0.2% acetic acid–acetonitrile–methanol (50:30:20); flow-rate, 1 ml/min; fraction collection, 0.18 ml per tube. Tol = taleranol; Zol = zeranol; Zone = zeralanone.

[11]. The radioactivity is distributed between three compounds (85%), the retention times of which correspond to those of standard taleranol, zeranol and zeralanone, plus a weakly retained and more polar compound that could correspond to conjugates. Confirmation of the identity of these metabolites was obtained from GC–MS analysis of the silylated derivatives. The supposed taleranol and zeranol gave identical spectra with [M′] = [M − 3TMS] = 538, [M′ − 15] = 523, [M′ − OTMS − 15] = 433 and [M − 15] = 307, which accords with recently published data [12]. The third metabolite exhibited [M + 2TMS] = 464, [M + 2TMS − 15] = 449 and [M − 15] = 307, which corresponds to trimethylsilylated zeralanone [12]. The quantitative distribution of the three free metabolites was taleranol 2.5%, zeranol 72.6% and zeralanone 24.9%.

The residual plasma radioactivity (91%) was analysed directly following ethanol deproteination and Sep-Pak C_{18} clean-up. These preliminary steps led to a 25% recovery of the initial plasma radioactivity. Using control plasma spiked with similar amounts of zeranol conjugates isolated from the bile of rats dosed with [^{3}H]zeranol [3], the recovery reached 85%. According to previous studies which showed that the steroid conjugates were carried over by transport proteins [13], it can be hypothesized that the limited extraction yield could result from interactions of proteins and zeranol conjugates. As zeranol conjugates have been described as major biliary and urinary excretion products in different species [3], an HPLC analysis was designed based on a reversed-phase column and ion-pairing, according to previous studies on the separation of steroid glucoronides using ion-exchange layers generated by a hydrophobic mobile phase containing an alkylammonium surfactant [14]. The radio-HPLC profile obtained is presented in Fig. 3. Four main compounds or groups of compounds were separated. A weakly retained fraction (A) represented 20% of the radioactivity and the three others accounted for 9.4, 30.7 and 39.5%, respectively. Fractions B, C and D were collected separately, then hydrolysed using helicase. HPLC analysis of the free metabolites indicated they had similar retention times to taleranol, zeranol and zeralanone, respectively. Their identity was confirmed using GC–MS as described. Therefore, it can be concluded that glucuro-conjugation represents a major metabolic pathway of zeranol in pigs.

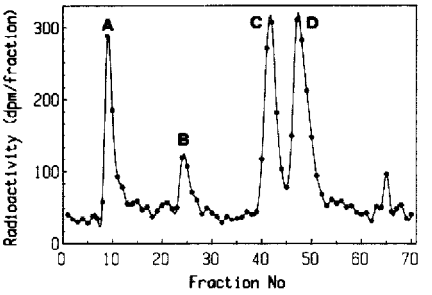

Fig. 3. HPLC metabolic profile of polar metabolites of [^{3}H]zeranol in pig plasma. Chromatographic conditions: Spherisorb ODS-1 C_{18} (5 μm) column (250 mm×0.4 mm I.D.); mobile phase, aqueous tetrabutylammonium solution (2.22 g/l, pH 7.2)-methanol (60:40); flow-rate, 1 ml/min; fraction collection, 0.18 ml per tube.

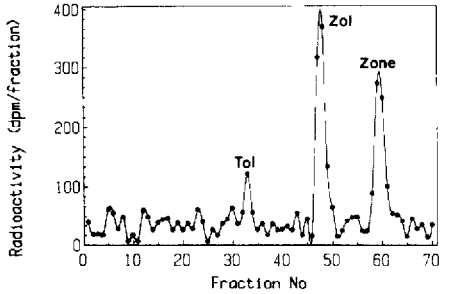

Fig. 4. HPLC metabolic profile of metabolites of [^{3}H]zeranol resulting from helicase enzymatic hydrolysis. Chromatographic conditions as in Fig. 2. Tol = taleranol; Zol = zeranol; Zone = zeralanone.

The enzymatic (helicase) analysis of the plasma, following prior solvent extraction of the free metabolites, led to an 81.8±15.0% (n=5) recovery of the residual radioactivity as extractable compounds. The HPLC analysis of these metabolites is shown in Fig. 4. Under chromatographic conditions identical with those used to separate the free metabolites, three very major peaks appeared that represent 89.2% of the radioactivity extracted. The remainder was distributed between different metabolites, none of them representing more than 4% of the total radioactivity of the extract. The retention times of the major compounds were identical with those of taleranol, zeranol and zeralanone and their relative abundances were 9.1, 42.7 and 37.4%, respectively. It should be noted that the 20% unaccounted for in the non-extractable metabolites from the original plasma disappeared under the action of helicase, whereas they resisted bacterial glucuronidase attack (data not shown). This suggests that these metabolites could be sulpho conjugates and gives additional support to the hypothesis already put forward by other workers [3].

CONCLUSION

These preliminary results indicate that zeranol is metabolized in the pig following similar pathways to those in the other species tested, i.e., oxido–reduction

to zeralanone and taleranol, then glucuro and sulpho conjugation as the major metabolic routes. The combination of the analytical approaches developed for this study is a powerful technique for an extended investigation of the in vivo metabolic fate of [^{3}H]zeranol.

ACKNOWLEDGEMENT

The authors are greatful to J.F. Sutra for skillful technical assistance.

REFERENCES

1 L.E. Denzer, L.H. Thompson, F.K. McKeith, D.E. Parrett and D.L. Thomas, J. Anim. Sci., 62 (1986) 1164.
2 R.S. Baldwin, R.D. Williams and M.K. Terry, Regul. Toxicol. Pharmacol., 3 (1983) 9.
3 B.H. Migdalof, H.A. Dugger, J.G. Heider, R.A. Coombs and M.K. Terry, Xenobiotica, 13 (1983) 209.
4 D.G. Sharp and I.A. Dyer, J. Anim. Sci., 34 (1972) 176.
5 G.H. Ingerowski and H.-J. Stan, J. Environ. Pathol. Toxicol., 2 (1970) 1173.
6 R. Verbeke, J. Chromatogr., 177 (1979) 69.
7 S.A. Dixon, J. Vet. Pharmacol. Ther., 3 (1980) 177.
8 S.N. Dixon and C.B. Mallison, J. Vet. Pharmacol. Ther., 6 (1983) 173.
9 S.N. Dixon and C.B. Mallison, J. Vet. Pharmacol. Ther., 9 (1986) 94.
10 J.E. Roybal, R.K. Mums, W.J. Morris, J.A. Hurlbut and W. Shimoda, J. Assoc. Off. Anal. Chem., 71 (1988) 263.
11 H.L. Kim, A.C. Ray and R.D. Stipanovic, J. Agric. Food Chem., 34 (1986) 312.
12 T.R. Covey, D. Silvestre, M.K. Hoffman and J.D. Henion, Biomed. Mass Spectrom., 15 (1988) 45.
13 M.C. Lebean and E.E. Beaulieu, J. Clin. Endocrinol. Metab., 30 (1970) 166.
14 S. van der Wal and J.F.K. Huber, J. Chromatogr., 149 (1978) 431.