THE IN VIVO HUMAN METABOLISM OF TIBOLONE


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ABSTRACT:

In vivo metabolism of tibolone was studied in three healthy postmenopausal volunteers after daily oral administration of 2.5 mg of tibolone for 5 days and a single dose of 2.5 mg = 555 kBq of [14C]tibolone on day 6. The 0- to 192-h recovery of radioactivity in urine and feces was 31.2 ± 10.5 and 53.7 ± 5.1%, respectively. Total 0- to 192-h recovery ranged from 78.5 to 94.2% of the dose and averaged 84.9%. Metabolites were putatively identified using high-pressure liquid chromatography in plasma, urine, and feces. The most important phase I metabolic reactions were reduction of the 3-keto group to 3α- and 3β-hydroxy metabolites, a shift of the Δ5(10)-double bond to a Δ4(9)-double bond, a reduction of the Δ4(9)-double bond to 5α,10-dihydro or 5β,10-dihydro metabolites, and hydroxylation at C2 and C7. The most important phase II metabolic reaction is sulfation of the C17 hydroxy group of tibolone and sulfation of the C3 hydroxy groups. In the circulation, over 75% of tibolone and its metabolites are present in the sulfated form. Local metabolism and local sulfatases may contribute to the tissue-specific activity. Using human microsomes, tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone appeared to be at least 50-fold less potent inhibitors of CYP1A2, CYP2C9, CYP3A4, and CYP3A4 compared with enzyme-selective inhibitors. Tibolone and its metabolites, therefore, are not likely to play a clinically significant role at the level of these cytochrome P450 enzymes with regard to the metabolism of coadministered drugs.

Tibolone [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one] is a tissue-specific compound with favorable effects on bone, vagina, climacteric symptoms, mood, and sexual well being in postmenopausal women. It has not been observed to stimulate the endometrium (Moore, 1999) or the breast, as demonstrated by the lower incidence of breast tenderness and lower mammographic density (Valdivia and Ortega, 2000). Therefore, in some tissues, tibolone has different effects than estrogens.

The metabolism of tibolone has been studied in female rats, rabbits, and dogs. A considerable number of metabolites were identified in this study using 1H NMR and mass spectroscopy, and qualitative and quantitative differences between species were observed (Jacobs et al., 1992; Verhoeven et al., 2002). Major phase I metabolic routes were the reduction of 3-keto to 3α- and 3β-hydroxy moieties, and the major phase II metabolic route was sulfate conjugation of the hydroxy groups at C3 and C17. Profiling of the target organs showed a tissue-specific distribution of metabolites. The majority of these metabolites existed as sulfate conjugates. These data in animals indicate that tibolone exerts its tissue-specific activities, at least partly, due to its tissue-specific metabolism and distribution. In addition, the presence of local sulfatases may convert inactive sulfated metabolites to active forms.

The linearity of the pharmacokinetic profile of tibolone was studied in three groups of nine healthy female volunteers using 1.25, 2.5, and 5 mg of tibolone, respectively. The pharmacokinetic profile was mainly based on the primary phase I plasma metabolites (i.e., tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone). The steady state was attained by day 5 in all three dose groups. Since in most cases the plasma concentration of tibolone and Δ4-tibolone was below the detection limit, their elimination half-life could not reliably be determined. The geometric mean value of the elimination half-life of 3α-hydroxy tibolone for the three dose levels ranged from 7.2 to 8.5 h. The very low plasma concentrations of the parent compound and the even lower concentrations of the Δ4-isomer, in combination with the considerably higher concentrations of the 3α- and 3β-hydroxy metabolites, indicated that tibolone is extensively metabolized, predominantly by hydroxylation at C3.

Human biotransformation pathways need to be identified, and the possibility of metabolic or pharmacokinetic interactions occurring with coadministered compounds need to be addressed during the development of a drug. In vitro approaches are usually used to study these issues and to evaluate their potential clinical relevance. These in vitro studies generally focus on cytochrome P450, which is a collective term for a group of enzymes that play a critical role in the oxidative metabolism (phase I metabolism) of the majority of drugs (Guengerich and Turvy, 1991; Shimada et al., 1994).

The present study investigated the in vivo human metabolism of tibolone in postmenopausal volunteers under steady-state conditions using [14C]tibolone. In addition, the interaction of tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone with the cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2E1, and CYP3A4 was studied in human liver microsomes.

Materials and Methods

Chemicals and Reference Compounds. The following nonlabeled and labeled compounds were supplied by NV Organon (Oss, The Netherlands).
The in vivo human metabolism of tibolone

Tibolone (Org OD14), Δ^-tibolone (Org OM38), 3α-hydroxy tibolone (Org 4094), 3β-hydroxy tibolone (Org 30126), $^{14}$C]-tibolone, specific activity 6.2 MBq·mg$^{-1}$, labeled at the 7α-methyl substituent; its radiochemical purity was $\geq89\%$. 3β-[16-3H]-Hydroxy tibolone, specific activity 1.5 TBq·mmol$^{-1}$; its radiochemical purity was $\geq80\%$. 3α-[16-3H]-Hydroxy tibolone, specific activity 1.5 TBq·mmol$^{-1}$; its radiochemical purity was $\geq80\%$. 3α,17β-Diisulfate tibolone, 3α-hydroxy-17β-sulfate tibolone, 3α-sulfate tibolone, isolated from animal studies and identified by mass spectrometry and NMR (Jacobs et al., 1992; Verhoeven et al., 2002), and Org OD14, Org OM38, Org 4094, and Org 30126 were used as reference compounds for profiling purposes.

Other reagents and solvents were obtained from commercial sources. They were of analytical grade and were used without further purification.

### Clinical Investigator and Study Center

The clinical part of this study was performed under the supervision of the principal investigator, S. P. van Marle, in the clinical research center of Pharma Bio-Research International BV (Zuidlaren, The Netherlands). The Local Ethical Committee approved the study protocol, and each subject gave her written informed consent before participation. The study was conducted in compliance with the Declaration of Helsinki and with Good Clinical Practice.

### Study Design, Dosing and Sample Collection

This was an open-label, multiple-dose study with no blinding procedures. Three healthy postmenopausal female subjects received a capsule of 2.5 mg of nonlabeled tibolone once daily for 5 consecutive days to attain steady-state plasma concentrations. Pausal female subjects received a capsule of 2.5 mg of nonlabeled tibolone at home until the concentration of radioactivity was below 1.25 Bq/400 mg of tissue. The volunteers were discharged from the clinic (last sample taken at 264 h). Plasma was prepared by centrifugation.

Urine samples were collected predose and in 8-h portions during the first 24 h after the radioactive dose and in 24-h portions thereafter, until discharged from the clinic (last sample taken at 264 h). Plasma was prepared by centrifugation.

Fecal samples were collected predose and in 8-h portions during the first 24 h after the radioactive dose and in 24-h portions thereafter, until the concentration of radioactivity (determined by “quick count”) was below 0.8 Bq·ml$^{-1}$, which was reached after 192 h or 240 h.

Based on data obtained with tibolone in animals, it was anticipated that the study would finish on the morning of day 13. Therefore, from day 6, excretion of radioactivity in urine and feces was examined on a daily basis by quick counts to follow the elimination of radioactivity. The volunteers were discharged from the clinic on day 17 of the study and continued to collect feces at home until the concentration of radioactivity was below 1.25 Bq/400 mg of homogenized feces.

Plasma, urine, and feces samples to be analyzed for metabolite profiling were stored at $\leq-20^\circ$C until shipment. Samples were shipped in a deep-frozen condition to NV Organon. After arrival, they were stored at $\leq-20^\circ$C until analysis.

### Determination of Radioactivity Concentrations

The concentration of radioactivity in plasma and urine was determined by liquid scintillation counting using a type Tri-Carb 2500 TR/2 Canberra Packard liquid scintillation counter (Packard Instrument Co., Meriden, CT).

The concentration of radioactivity in feces was determined by combustion in a type 387 Canberra Packard sample oxidizer, followed by liquid scintillation counting. Feces samples were homogenized with approximately 2 volumes of Milli-Q water (Millipore Corporation, Bedford, MA) before combustion.

### Pharmacokinetics

The area under the total plasma concentration versus time curves (AUC) were determined by the trapezoidal rule. The half-life of elimination of radioactivity was obtained as follows: from visual inspection of the individual log-concentration versus time plots, it was determined from which time point the plot was approximately linear. Using log-linear regression on these terminal data points of the concentration-time curve, the elimination half-life was calculated.

### Pooling of Samples for Metabolite Profiling

For metabolite profiling, plasma samples containing sufficient radioactivity were pooled per sampling time point (1, 1.5, 2, 3, 4, 6, 8, 24, and 48 h after dosing). The plasma samples of later time points did not contain enough radioactivity for the purpose of metabolite profiling. For urine and feces samples, samples containing at least 1.7% of the administered dose were selected for metabolite profiling. This resulted in analyzed urine and feces samples representing 70 to 86% of the administered dose.

### Sample Treatment for the Analysis of Metabolite Profiles

Selected biological fluids, containing an adequate amount of radioactivity, were concentrated and then profiled by direct injection on the HPLC column. These profiles were qualitatively and quantitatively compared with the corresponding extracted samples. In case of no significant differences (determined visually), the remaining samples were analyzed for practical reasons by pretreatment procedures. Plasma proteins were precipitated by the addition of ice-cold acetone. After centrifugation, the supernatant was concentrated by vacuum centrifugation and subjected to HPLC analysis. The extraction recovery of radioactivity was 62 ± 9%.

Urine was applied to 6-ml pretreated Bakerbond SPE C$_{18}$ solid-phase extraction columns. Columns were washed with ammonium acetate (0.1 M, pH 4.2) and eluted with methanol. The methanol eluents were dried by vacuum centrifugation; residues were dissolved in Milli-Q water and subjected to HPLC analysis. The extraction recovery of radioactivity was 101 ± 26%.

Feces samples were extracted with 1.5 volumes of acetonitrile (extraction recovery of radioactivity, 72 ± 13%). The extracts were dried by vacuum centrifugation; residues were taken up in methanol/Milli-Q water and applied to 6-ml pretreated Bakerbond SPE C$_{18}$ solid-phase extraction columns. Columns and methanol eluents were treated as described for urine samples, except that the residues were dissolved in a small volume of methanol instead of Milli-Q water. The solid phase extraction recovery of radioactivity was 97 ± 9%.

### HPLC Analysis of Metabolite Profiles in Plasma, Urine, and Feces

HPLC analysis of the plasma, urine, and feces samples was performed using a $\mu$Bondapak C$_{18}$ column (internal diameter, 3.9 mm; internal length, 300 mm) and a gradient of ammonium acetate buffer (0.1 M, pH 4.2) (solvent A) and methanol (solvent B). Elution was performed with a linear gradient of 25 to 90% solvent B (v/v) for 20 min at 50°C. The flow rate was 1.7 ml/min. HPLC analysis was performed with a type HP1090 liquid chromatograph equipped with a type HP1040 diode array detector (Hewlett Packard, Waldbronn, Germany). Radioactivity in the HPLC effluent was determined on-line using a type A525 flow-through Flo-One beta radioactivity detector or by the collection of fractions followed by liquid scintillation counting. Samples were spiked with unlabeled Δ^-tibolone before HPLC analysis as an internal reference for the retention time (UV signal at 254 nm). Reference compounds (isolated or authentic synthesized) were analyzed for their retention times in “in-between” runs. Quantification of tibolone and Δ^-tibolone is not possible by the described HPLC method. Tibolone is not stable enough under applied conditions and will isomerize into Δ^-tibolone. No attempts have been made to correct for this phenomenon.

### Inhibition study

**Microsomes.** Human liver microsomes from different organ donors were supplied by Human Biologics, Inc. (Phoenix, AZ). Human Biologics, Inc. fully complies with all applicable laws governing the sale of processed human biomaterials for commercial research, including the Uniform Anatomical Gift Act. The cytochrome P450 content of the pooled microsomal preparation was 0.48 nmol/mg of protein (data from Human Biologics, Inc.).

**Test compounds and model inhibitors.** Model inhibitors, fluoroxamine, and ketocazole (selective inhibitors for CYP1A2 and CYP3A4, respectively) were obtained from Dispensing Services and Control, NV Organon. [14C]-testosterone, to be used as a substrate for CYP3A incubations, was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Model inhibitors, sulfaphenazole, and diethyldithiocarbamate (selective inhibitors for CYP2C9 and CYP2E1, respectively) were obtained from Ultrafine Chemicals (Manchester, UK) and Aldrich Chemie (Zwijndrecht, The Netherlands), respectively.
TABLE 1

Percentage of the dose excreted in the urine and feces of postmenopausal volunteers after oral administration of [14C]tibolone

Data are expressed as mean ± S.D. (n = 3 subjects).

<table>
<thead>
<tr>
<th>Time Intervala</th>
<th>Radioactivity in Urine and Feces</th>
<th>% of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–8</td>
<td>10.0 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>8–16</td>
<td>4.0 ± 2.0</td>
<td></td>
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<tr>
<td>16–24</td>
<td>2.6 ± 0.5</td>
<td>1.0 ± 1.7</td>
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<tr>
<td>24–48</td>
<td>6.1 ± 2.6</td>
<td>6.3 ± 8.2</td>
</tr>
<tr>
<td>48–72</td>
<td>3.6 ± 1.8</td>
<td>15.7 ± 2.6</td>
</tr>
<tr>
<td>72–96</td>
<td>2.1 ± 1.0</td>
<td>21.7 ± 10.5</td>
</tr>
<tr>
<td>96–120</td>
<td>1.2 ± 0.6</td>
<td>1.7 ± 3.0</td>
</tr>
<tr>
<td>120–144</td>
<td>0.6 ± 0.2</td>
<td>4.9 ± 1.6</td>
</tr>
<tr>
<td>144–168</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>168–192</td>
<td>0.4 ± 0.1</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Total 0–192</td>
<td>31.2 ± 10.5</td>
<td>53.7 ± 5.1</td>
</tr>
<tr>
<td>Total 0–192</td>
<td>84.8 ± 8.3</td>
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</tbody>
</table>

(Urine + Feces)

a Time post radioactive dose.

b Feces: 0- to 24-h fraction instead of 16- to 24-h fraction.

Incubation Experiments. The study consisted of three types of incubations involving a pool of human liver microsomes and 1) the enzyme-selective substrates, 7-ethoxyresorufin (Burke and Mayer, 1974; Dutton and Parkinson, 1989), diclofenac (Leemann et al., 1992), chlorozoxane (Peter et al., 1990) (Sigma Chemical Co., St. Louis, MO), and testosterone (Brian et al., 1990); 2) the enzyme-selective substrates in the presence of Org OD14, Org 4094, Org 30126, or Org OM38; or 3) the enzyme-selective substrates in the presence of model inhibitors known to be selective for each of the human cytochrome P450 enzymes.

Each substrate was incubated at seven different concentrations. Org OD14 (50 and 500 μM), Org 4094 (10, 25, 50, and 500 μM), Org 30126 (25, 50, and 500 μM) and Org OM38 (50, 200, and 500 μM) were incubated at two to four different concentrations. The model inhibitors fluvoxamine (0.1 and 0.5 μM), sulfaphenazole (0.5 and 1.5 μM), diethylthiocarbamate (5 and 50 μM), and ketoconazole (0.05 and 0.2 μM) were incubated at two different concentrations. The incubation mixtures contained 3 mM NADPH. Blanks were incubations without NADPH.

Data Analysis. For Org OD14, Org 4094, Org 30126, Org OM38, and each of the model inhibitors, Ki values were determined with the curve-fitting program for the analysis of enzyme kinetic data called “EZ-FIT” (Perella, 1988). The type of inhibition was established by means of Hanes-Woolf plots.

Results

Excretion of Radioactivity in Urine and Feces. Data on the excretion of radioactivity in urine and feces (0–192 h) are given in Table 1. Urine was sampled up to at least 192 h after the radioactive dose. The recovery of radioactivity in urine over the 0- to 192-h interval ranged from 19.7 to 40.4% and averaged 31.2 ± 10.5%.

Feces samples were obtained up to 336 or 360 h after the radioactive dose. For the time interval of sampling both urine and feces (0–192 h), the recovery of radioactivity in feces ranged from 48.6 to 58.8%, with a mean value of 53.7 ± 5.1% of the dose. Little further excretion occurred; the 0- to 336-h recovery of radioactivity in feces averaged 55.7% of the dose. The total recovery of radioactivity within 192 h after the radioactive dose averaged 84.8 ± 8.3%.

Radioactivity in Plasma. Radioactivity appeared in plasma within 1 h after the radioactive dose. Peak concentrations were reached 3 h after the dose (Table 2). The terminal half-life of radioactivity was 129, 121, and 123 h for subjects 1, 2, and 3, respectively.

HPLC Metabolite Profiles in Plasma. The metabolite profiles in pooled plasma samples obtained from postmenopausal female volunteers after daily oral administration of 2.5 mg of tibolone for 5 days, followed by a single oral dose of 2.5 mg of [14C]tibolone, are included in Table 2. Tibolone and its metabolites were quantified as nanograms of equivalents per milliliter of plasma.

On the basis of the retention times of metabolites isolated and identified in animal studies and the retention times of the reference compounds used, putatively identified compounds included 3α,17β-disulfate tibolone, 3α-hydroxy-17β-sulfate tibolone, 3α-sulfate tibolone, 3α-hydroxy tibolone, and Δ4-tibolone. These compounds were major plasma metabolites, as indicated by the calculated AUC values (Table 2). Over 75% of tibolone and its metabolites are present in the circulation in the sulfated form. The quantification of Δ4-tibolone, as presented in Table 2, might be overestimated, and consequently, the tibolone concentration might be underestimated. It appeared that under the applied extraction procedure, tibolone itself could isomerize into Δ4-tibolone. This was found to be a chemical process occurring under acidic conditions. In this study, no attempts have been made to correct for this isomerization process.

HPLC Metabolite Profiles in Urine and Feces. Metabolite profiles of the urine and feces samples obtained from the three postmenopausal volunteers after oral administration of [14C]tibolone are given in Tables 3 and 4 for urine and feces, respectively. Tibolone and its metabolites were quantified as a percentage of the radioactive dose.

Sixteen compounds were found in the urine (designated as U1 to U16) and nineteen compounds in the feces (designated as F1 to F19). Some of the compounds found in urine and feces coeluted in pairs in many chromatograms (U1-U4, U7-U8, F3-F4, F14-F15, and F16-F19). Furthermore, matrix influences caused small drifts in the retention time values, as determined with isolated metabolites (U9-U10, U11-U12, U13-U14, and F8-F10). Corrections were made based on the relative retention time indices. The absolute ranges for the individual chromatograms are given in Tables 3 and 4. For mean calculations, some peaks were taken together. This has been indicated in Tables 3 and 4.

On the basis of the retention times of metabolites isolated and identified in animal studies and the retention times of the reference compounds used, the main urinary metabolites were putatively identified as 3α,17β-disulfate tibolone (U9-U10), 3α-hydroxy-17β-sulfate tibolone (U11-U12), and 3α-sulfate tibolone (U13-U14), respectively. Metabolite U5 was tentatively characterized as 3α,7β-dihydroxy-5α,10β-dihydro-17β-sulfate tibolone and U6 as 7β-hydroxy-3α-sulfate tibolone. The characterized compounds excreted with urine accounted for approximately 20% of the total dose that was administered. The unidentified 5.8% were characterized as conjugated (most probably sulfated) metabolites, based on their retention time profile in the HPLC analyses.

The following compounds were putatively identified in feces: 3α,17β-disulfate tibolone (F5), 3α-hydroxy-17β-sulfate tibolone (F7), 3α-sulfate tibolone (F8-F10), and Δ4-tibolone (F12). Metabolite F1 was tentatively characterized as 3α,7β-dihydroxy-5α,10β-dihydro-17β-sulfate tibolone, F2 as 7β-hydroxy-3α-sulfate tibolone, and F6 as 2α-hydroxy-3α-sulfate tibolone. The latter compound was not found in urine. F13 was tentatively identified as 3α-hydroxy tibolone and F14-F15 as 3β-hydroxy tibolone or tibolone. The latter two compounds could not be separated under the applied conditions. The characterized compounds excreted with feces accounted for 50% of the total radioactive dose. Of the remaining 10%, approximately 5% were characterized as conjugated (most probably sulfated) metabolites, based on the retention time profile in the HPLC analyses.

For the same reason as given for plasma, the quantification of Δ4-tibolone presented in Tables 3 and 4 might be overestimated, and consequently, the tibolone concentration might be underestimated.
appeared that under the applied extraction procedure, tibolone itself could isomerize into $\Delta^4$-tibolone.

Based on the metabolites identified in the present study, the proposed biotransformation of tibolone in postmenopausal volunteers is given in Fig. 1. The overview of excreted metabolites and exposure data given in Table 5 shows that the majority of the metabolites are present as sulfates (72% of total dose) and have the 3α-configuration (66% of total dose).

**Inhibition Study.** $K_i$ values and the type of inhibition (competitive or noncompetitive) were determined for tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and each of the model inhibitors (Table 6). The type of inhibition was determined on the basis of Hanes-Woolf plots.

Fluvoxamine was a noncompetitive inhibitor of CYP1A2 with a $K_i$ value of 0.21 $\mu$M. Tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and $\Delta^4$-tibolone had only minor effects on CYP1A2 activity. The four test compounds were tested at a concentration of 500 $\mu$M. During the study, it was found that this concentration was above the

$\Delta^4$-configuration.
postmenopausal women is the reduction of the 3-keto group to 3α- and 3β-hydroxy metabolites, a reaction catalyzed by 3α-hydroxy-steroid-dehydrogenase/isomerase (HSD) and 3β-HSD, respectively. Other phase I reactions are a shift of the 5(10) double bond to a 4(5) double bond catalyzed by Δ5-3-isomerase, reduction of the 4(5) double bond to 5α,10β-dihydro or 5β,10β-dihydro metabolites catalyzed by 5α-reductase, and hydroxylation at C2 and C7 catalyzed by cytochrome P450. The most important phase II metabolic reaction is sulfation of the C3 hydroxy groups formed during phase I metabolism and the C17 hydroxyl group of tibolone. The presence of sulfatases that are able to locally convert the inactive sulfated metabolites to active metabolites may contribute to the tissue-specific effects of tibolone. In addition, tibolone, in contrast to estrogens, inhibits sulfatase activity in the breast, resulting in reduced formation of estradiol from estrone sulfate in breast tissue (Pasqualini and Chetrite, 1999).

To obtain information on the effect of tibolone on the metabolism/pharmacokinetic profile of a coadministered compound, the inhibition of cytochrome P450 enzymes by tibolone and its major phase I metabolites was studied in vitro. Tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone appeared to be weak inhibitors of CYP1A2, CYP2C9, CYP2E1, and CYP3A4 in comparison to the enzyme-selective inhibitors used in this study.

The extent of in vivo inhibition by tibolone and its metabolites can be predicted as:

\[
\% \text{ inhibition} = \frac{[I]}{[S] + K_i (1 + [S]/K_m)} \cdot 100 \quad \text{(competitive inhibition)}
\]

where [I] is the concentration of inhibitor, \(K_i\) is the inhibition constant, [S] is the concentration of substrate, and \(K_m\) is the Michaelis-Menten constant (Segel, 1975). Since the local concentrations of drug and inhibitor are difficult to determine, the in vivo plasma concentrations are often used to approximate the concentration at the site of potential metabolic interaction.

Since the peak plasma concentration of most drugs is far below their \(K_m\), the contribution of \([S]/K_m\) can generally be neglected, and the equation for the prediction of the extent of in vivo inhibition becomes:

\[
\% \text{ inhibition} = \frac{[I]}{[I] + K_i} \cdot 100
\]

which is identical with the equation for prediction of the in vivo inhibition for noncompetitive inhibitors (Segel, 1975).

In a pharmacokinetic multiple dose study using the maximum dose of tibolone for clinical use (2.5 mg/day), mean peak plasma concentrations at steady state were 1.7, 14.2, 3.8, and 0.4 ng/ml (5, 50, 12, and 1.3 nM) for tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone, respectively. The extent of in vivo inhibition of the metabolism of coadministered drugs would be expected to be highest for CYP3A and 3α-hydroxy tibolone/3β-hydroxy tibolone, for which the lowest \(K_i\) values were observed in combination with the highest mean peak plasma concentrations at steady state. The extent of in vivo inhibition of the metabolism of substrates of CYP3A is predicted to be 1.4 and 0.2% for 3α-hydroxy tibolone and 3β-hydroxy tibolone, respectively. Assuming a 10-fold higher concentration in the liver compared with plasma, the in vivo inhibition would be predicted to be less than 12 and 1.9% for 3α-hydroxy tibolone and 3β-hydroxy tibolone, respectively. From this study, it can be concluded that tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-isomer are not likely to display a clinically significant inhibition at the level of CYP1A2, CYP2C9, CYP2E1, and CYP3A4 with regard to the metabolism of coadministered drugs.

**Discussion**

The in vivo human results presented in this article confirm the metabolic routes previously shown in in vitro studies (Sandker et al., 1994) and in vivo in rat, rabbit, and dog (Verhoeven et al., 2002). The most important phase I metabolic reaction found for tibolone in

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**FIG. 1. Summary of the metabolic routes of tibolone in postmenopausal women.**

- **A**, 2α-hydroxylation; **B**, reduction of 3-keto moiety to a 3α-hydroxy group, followed by sulfation or reduction to a 3β-hydroxy group; **C**, isomerization; **D**, reduction of the Δ5,10-double bond; **E**, 7β-hydroxylation; **F**, sulfation of the 17β-hydroxy group.

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level of maximum solubility under the conditions used. A pilot experiment performed at concentrations of 50, 100, 200, and 500 μM indicated that tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone were completely dissolved at 50, 50, 200, and 100 μM, respectively. \(K_i\) values were calculated using these concentrations and were presumably somewhat underestimated. The data indicated that tibolone and its metabolites were at least 500-fold less potent inhibitors of CYP1A2 than fluvoxamine.

Sulfaphenazole was a competitive inhibitor of CYP2C9 with a \(K_i\) value of 0.28 μM. Tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone were also competitive inhibitors of CYP2C9. The respective \(K_i\) values were 14.8, 17.4, 84.2, and 32.9 μM, indicating that tibolone and its metabolites are at least 50-fold less potent inhibitors than sulfaphenazole. Diethyldithiocarbamate was a noncompetitive inhibitor of CYP2E1, with a \(K_i\) value of 33.0 μM. Tibolone, 3α-hydroxy tibolone, 3β-hydroxy tibolone, and Δ4-tibolone did not inhibit CYP2E1 at maximum solubility under the conditions used.

In the present study, the inhibition of CYP3A4 displayed by ketoconazole showed the best fit with the competitive inhibition model. The \(K_i\) values for CYP3A4 were obtained using the competitive model and were 0.017 and 0.02 μM for 0.05 and 0.4 μM ketoconazole, respectively. Tibolone, 3α-hydroxy tibolone, and 3β-hydroxy tibolone were competitive inhibitors and Δ4-tibolone was a noncompetitive inhibitor of CYP3A4. The respective \(K_i\) values were 14.5, 3.57, 6.05, and 62.8 μM. The data obtained indicate that ketoconazole inhibits CYP3A4 at least a 125-fold more potently than tibolone and its metabolites.
Generally, in vitro studies include the characterization of the cytochrome P450 enzymes involved in the primary metabolic routes of the drug. We concluded that these data for tibolone would be irrelevant for the clinical situation because tibolone is transformed in vivo very rapidly by the 3α-HSD and 3β-HSD enzymes. Oxidation by CYP450 enzymes presumably occurs thereafter and competes with sulfation by the sulfotransferases. The in vitro CYP450 microsomal studies with tibolone cannot mimic this in vivo situation. Incubations with 3α- and 3β-hydroxy tibolone should offer a more relevant alternative. However, because it is not clear which metabolite forms the predecessor for oxidation at C2 or C7 and because less than 12% of the metabolites are oxidized at position C2 or C7, we concluded that this study would not provide the relevant information.

C2 and C7 hydroxylation are the only phase I metabolic reactions catalyzed by cytochrome P450 and are only minor pathways in quantitative terms. Consequently, it is unlikely that inhibition or induction of cytochrome P450 by coadministered compounds will affect the metabolic elimination of tibolone. Furthermore, tibolone is metabolized by multiple pathways, indicating that other routes of biotransformation may compensate for interactions at one of these pathways. To our knowledge, interactions at the level of 3α- or 3β-HSD or Δ4,5-isomerase have never been reported.

In conclusion, our data show that the metabolism of tibolone in the presence of ketoconazole has a sigmoidal character, which could be described by the competitive inhibition model. However, it is not clear which metabolite forms the predecessor for oxidation at C2 or C7 and because less than 12% of the metabolites are oxidized at position C2 or C7, we concluded that this study would not provide the relevant information.

### Acknowledgments

We thank Dr. S. P. van Marle for the clinical portion of the study, H. M. van den Wildenberg for the LC-MS analyses, Dr. J. Boogaards (TNO) for performing the inhibition stud-

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TABLE 5
Overview of excretion data and plasma exposure data

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Dose</th>
<th>Plasma AUC_{0-24 h}</th>
<th>ngeq \cdot h \cdot ml^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Total^a</td>
</tr>
<tr>
<td>3α,7β-Dihydroxy-5α,10β-dihydro-17β-sulfate tibolone</td>
<td>3.97</td>
<td>0.77</td>
<td>5</td>
</tr>
<tr>
<td>7β-Hydroxy-3α-sulfate tibolone</td>
<td>1.49</td>
<td>1.04</td>
<td>3</td>
</tr>
<tr>
<td>3α,17β-Di-Sulfate tibolone</td>
<td>7.05</td>
<td>5.17</td>
<td>12</td>
</tr>
<tr>
<td>2α-Hydroxy-3α-sulfate tibolone</td>
<td>0</td>
<td>4.01</td>
<td>4</td>
</tr>
<tr>
<td>3α-Hydroxy-17β-sulfate tibolone</td>
<td>2.77</td>
<td>8.81</td>
<td>12</td>
</tr>
<tr>
<td>3α-Sulfate tibolone</td>
<td>4.03</td>
<td>13.27</td>
<td>17</td>
</tr>
<tr>
<td>Δ4,5-Tibolone</td>
<td>0.65</td>
<td>8.39</td>
<td>9</td>
</tr>
<tr>
<td>3α-Hydroxy tibolone</td>
<td>0</td>
<td>3.83</td>
<td>4</td>
</tr>
<tr>
<td>3β-Hydroxy tibolone/17β-dihydro-17β-sulfate tibolone</td>
<td>5.83</td>
<td>4.37</td>
<td>10</td>
</tr>
<tr>
<td>Remainder conjugated</td>
<td>0.01</td>
<td>4.61</td>
<td>5</td>
</tr>
<tr>
<td>Remainder unconjugated</td>
<td></td>
<td></td>
<td>37</td>
</tr>
</tbody>
</table>

^a Data are rounded off.

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TABLE 6
Inhibition of human cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2E1, and CYP3A by Org OD14, Org 4094, Org 30126, Org OM38, and enzyme-selective inhibitors^b

Results are presented as means ± S.D.

<table>
<thead>
<tr>
<th>Cytochrome P450</th>
<th>Inhibitor</th>
<th>Type of Inhibition</th>
<th>$K_{in}$ (μM)</th>
<th>$V_{max}$ ^b (pmol/min/mg)</th>
<th>$K_I$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>None</td>
<td>None</td>
<td>0.25 ± 0.03</td>
<td>118 ± 4</td>
<td>100</td>
</tr>
<tr>
<td>Org OD14</td>
<td>Competitive</td>
<td>0.03 ± 0.04</td>
<td>126 ± 6</td>
<td>0.21 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Org 4094</td>
<td>Competitive</td>
<td>12.6 ± 0.8</td>
<td>3178 ± 65</td>
<td>14.8 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Org 30126</td>
<td>Competitive</td>
<td>12.2 ± 1.1</td>
<td>3081 ± 86</td>
<td>17.4 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Org OM38</td>
<td>Competitive</td>
<td>13.1 ± 0.9</td>
<td>3168 ± 64</td>
<td>84.2 ± 14.3</td>
<td></td>
</tr>
<tr>
<td>CYP29</td>
<td>None</td>
<td>Noncompetitive</td>
<td>238 ± 29</td>
<td>2047 ± 119</td>
<td>33.0 ± 2.6</td>
</tr>
<tr>
<td>Org OD14</td>
<td>Competitive</td>
<td>12.6 ± 1.2</td>
<td>3089 ± 94</td>
<td>14.8 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Org 4094</td>
<td>Competitive</td>
<td>12.2 ± 1.1</td>
<td>3081 ± 86</td>
<td>17.4 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Org 30126</td>
<td>Competitive</td>
<td>13.1 ± 0.9</td>
<td>3168 ± 64</td>
<td>84.2 ± 14.3</td>
<td></td>
</tr>
<tr>
<td>Org OM38</td>
<td>Competitive</td>
<td>12.8 ± 1.4</td>
<td>3256 ± 108</td>
<td>32.9 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>None</td>
<td>Noncompetitive</td>
<td>0.05 ± ketoconazole</td>
<td>64.0 ± 9.1</td>
<td>0.017 ± 0.003</td>
</tr>
<tr>
<td>Org OD14</td>
<td>Competitive</td>
<td>62.7 ± 8.1</td>
<td>9464 ± 393</td>
<td>14.5 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Org 4094</td>
<td>Competitive</td>
<td>61.8 ± 7.1</td>
<td>9474 ± 350</td>
<td>3.57 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>Org 30126</td>
<td>Competitive</td>
<td>63.3 ± 7.0</td>
<td>9557 ± 347</td>
<td>6.05 ± 0.67</td>
<td></td>
</tr>
<tr>
<td>Org OM38</td>
<td>Noncompetitive</td>
<td>56.3 ± 9.7</td>
<td>4727 ± 331</td>
<td>62.8 ± 8.8</td>
<td></td>
</tr>
<tr>
<td>0.05 μM ketoconazole</td>
<td>Competitive^b</td>
<td>64.0 ± 9.1</td>
<td>9560 ± 449</td>
<td>0.017 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>0.2 μM ketoconazole</td>
<td>Competitive^b</td>
<td>65.7 ± 8.3</td>
<td>9683 ± 410</td>
<td>0.020 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

^a $K_{in}$ and $V_{max}$ values given for incubations in the presence of Org OD14, Org 4095, Org 30126, Org OM38, or a model inhibitor represent back-calculated values in the EZ-FIT curve-fitting program and are only indicative of a goodness of fit.

^b The best fit for ketoconazole was obtained with the competitive inhibition model. However, data showed that the metabolism of testosterone in the presence of ketoconazole has a sigmoidal nature. Therefore, the type of CYP3A4 inhibition by ketoconazole is not clear.

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Notes:

- $K_{in}$ and $V_{max}$ values given for incubations in the presence of Org OD14, Org 4095, Org 30126, Org OM38, or a model inhibitor represent back-calculated values in the EZ-FIT curve-fitting program and are only indicative of a goodness of fit.

- The best fit for ketoconazole was obtained with the competitive inhibition model. However, data showed that the metabolism of testosterone in the presence of ketoconazole has a sigmoidal nature. Therefore, the type of CYP3A4 inhibition by ketoconazole is not clear.
ies, and Drs. H. P. Wijnand and H. A. M. Verheul for editorial support.

References


