Selective Tissue Distribution of Tibolone Metabolites in Mature Ovariectomized Female Cynomolgus Monkeys after Multiple Doses of Tibolone

H. A. M. Verheul, M. L. P. S. van Iersel, L. P. C. Delbressine, and H. J. Kloosterboer

Research and Development, NV Organon, Oss, The Netherlands

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ABSTRACT:
Tibolone is a selective tissue estrogenic activity regulator (STEAR). In postmenopausal women, it acts as an estrogen on brain, vagina, and bone, but not on endometrium and breast. Despite ample supporting in vitro data for tissue-selective actions, confirmative tissue levels of tibolone metabolites are not available. Therefore, we analyzed tibolone and metabolites in plasma and tissues from six ovariectomized cynomolgus monkeys that received tibolone (0.5 mg/kg/day by gavage) for 36 days and were necropsied at 1, 1.25, 2.25, 4, 6, and 24 h after the final dose. The plasma and tissue levels of active, nonsulfated (tibolone, 3\alpha-hydroxytibolone, and 3\alpha,17\beta-dihydroxytibolone, and Δ^4-tibolone), monosulfated (3α-sulfate,17\beta-hydroxytibolone and 3β-sulfate,17\beta-hydroxytibolone), and disulfated (3α,17\beta-disulfated-tibolone and 3β,17\beta-disulfated-tibolone) metabolites were measured by validated gas chromatography with mass spectrometry and liquid chromatography with tandem mass spectrometry. Detection limits were 0.1 to 0.5 ng/ml (plasma) and 0.5 to 2 ng/g (tissues). In brain tissues, estrogenic 3α-hydroxytibolone was predominant with 3 to 8 times higher levels than in plasma; levels of sulfated metabolites were low. In vaginal tissues, major nonsulfated metabolites were 3α-hydroxytibolone and the androgenic/progestagenic Δ^4-tibolone; disulfated metabolites were predominant. Remarkably high levels of monosulfated metabolites were found in the proximal vagina. In endometrium, myometrium, and mammary glands, levels of 3-hydroxymetabolites were low and those of sulfated metabolites were high (about 98% disulfated). Δ^4-Tibolone/3-hydroxytibolone ratios were 2 to 3 in endometrium, about equal in breast and proximal vagina, and 0.1 in plasma and brain. It is concluded that tibolone metabolites show a unique tissue-specific distribution pattern explaining the tissue effects in monkeys and the clinical effects in postmenopausal women.

Abbreviations

Tibolone (Livial, Organon, Roseland, NJ and Oss, The Netherlands) is used for the management of postmenopausal symptoms: it relieves hot flushes and vaginal dryness, improves mood and libido, and prevents osteoporosis (Kloosterboer, 2004; Kenemans and Sperboer, 2005; Landgren et al., 2005; Swanson et al., 2006). It does not act as an estrogen on endometrium, resulting in a low incidence of vaginal bleeding (Morris et al., 1999). It has little effects on breast as shown by no increase in mammographic density or tenderness (Lundström et al., 2002). Thus, it differs from estrogen-progestagen combinations. These effects of tibolone on different tissues are explained by the hypothesis that it is a selective tissue estrogenic activity regulator (STEAR).

In postmenopausal women, 3α-hydroxytibolone (3αOH-tib) is the predominant nonsulfated, free metabolite in blood, followed by 3β-hydroxytibolone (3βOH-tib) (for structures, see Fig. 1). Both 3-hydroxymetabolites bind to estrogen receptors (de Gooyer et al., 2003; Kloosterboer, 2004) and are responsible for the estrogenic activity. Serum levels of tibolone and the Δ^4-isomer of tibolone (Δ^4-tib), which bind to progestagenic and androgenic receptors (de Gooyer et al., 2003), are low and become undetectable after 4 to 6 h (Timmer and Houwing, 2002; Timmer et al., 2002). More than 75% of the metabolites in blood are sulfated (Vos et al., 2002). Tibolone and its metabolites have no antagonistic effect on estrogen receptors, rendering it different from selective estrogen receptor modulators (Kloosterboer, 2004). To explain why tibolone is estrogenic in some tissues but not in others, it was postulated that tibolone and its active metabolites are converted into sulfated metabolites and/or into the androgenic/progestagenic Δ^4-tib, as has been shown in vitro (Kloosterboer, 2004). Tibolone can be converted to Δ^4-tib in endometrial explants (Tang et al., 1993); enzymes of the aldoketoreductase family (AKR1C) are able to reduce tibolone to 3-hydroxymetabolites (Steckelbreck et al., 2006) and back again (Schatz et al., 2005). In addition, 3αOH-tib and 3βOH-tib are substrates for sulfotransferases (SULT) expressed in different tissues (Chetrite et al., 1999a; Falany et al., 2004); SULT2A1 has been reported to be the major endogenous enzyme responsible for sulfation of tibolone metabolites in postmenopausal human tissues (Wang et al., 2006). Although sulfated metabolites are not active at receptor levels, sulfatases are able to readily convert 3-mono- and 17-sulfated, steroids into active estrogenic metabolites (de Gooyer et al., 2001). In breast cell lines, tibolone and its nonsulfated and sulfated metabolites inhibit...
Materials and Methods

Study Design. A study in mature, healthy, female, ovariectomized cynomolgus monkeys (n = 6; weight 2.0–2.8 kg; supplied by Primate Products, Miami, FL) was approved by the Institutional Animal Care and Use Committee, performed at Huntingdon Life Sciences (East Millstone, NJ and Huntingdon, Cambridgeshire, UK), and complied with the Animal Welfare Act Regulations and Good Laboratory Practice standards as described elsewhere (Verheul et al., 2007). After at least 1 month postovariectomy, the monkeys received a single dose, and after a washout of 1 week, they received repeated daily p.o. doses of tibolone (Org OD14; 0.5 mg/kg/day) from days 8 through 44 via nasogastric gavage. Blood, urine, and feces were collected for 7 days after the doses on days 1 and 36 for the assessment of the pharmacokinetic parameters of the sulfated and nonsulfated tibolone metabolites. These results are reported elsewhere (Verheul et al., 2007). At day 44, one monkey was necropsied at each of the following times: 1, 1.25, 2.25, 4, 6, and 24 h after the final dose. This design was chosen to establish concentration-time curves in plasma and tissues, rather than to determine the concentrations at one time point in six animals. Blood was collected into tubes containing K$_3$EDTA and centrifuged for 10 min at 2000g within 1 h, and plasma was stored below −20°C until analysis. The following tissues were washed free of blood and stored at −20°C until further processing and analysis: brain (cortex, hypothalamus, brainstem, cerebellum, hippocampus, midbrain, corpus callosum, and corpus striatum), vagina (distal and proximal), uterus (endometrium and myometrium), breast (mammary glands and fat), and heart.

Assay Methods. The assay procedures for the determination of tibolone and its nonsulfated and sulfated metabolites in plasma and tissue homogenates have been described elsewhere (Verheul et al., 2007).

After weighing, tissues were homogenized with internal standards in 70% ethanol for 5 to 15 min using an ultraturrax. The homogenates were divided into aliquots and stored for at least 24 h before further sample processing. The aliquots were centrifuged; the supernatant was evaporated, redissolved in water, and analyzed. In one aliquot, tibolone, 3αOH-tib, and 3β-tib were determined by gas chromatographic and mass spectrometric procedures (ABL, Assen, The Netherlands). The second aliquot was subjected to protein precipitation for analysis of the di-S metabolites: 3αS,17β-tib and 3βS,17β-tib. Liquid-liquid extraction was performed on the third aliquot, Δ4-tib was determined in the organic phase; and the mono-S metabolites (3αS,17βOH-tib and 3βS,17βOH-tib) were extracted by solid-phase extraction from the water phase. All the analytes were determined by liquid chromatography followed by tandem mass spectrometry procedures (Xendo, Groningen, The Netherlands). It was decided to develop assays for the 3αS-monos metabolites only because 1) the supply of most tissues was very limited; 2) the levels of the 17β-sulfated and 3αS,17β-tib metabolites were shown in a pilot experiment to be very low in myometrium and liver; and 3) the 17β-monos metabolites cannot readily be reconverted to the nonsulfated metabolites again (Goldzieher et al., 1988; de Gooyer et al., 2001; Takahashi et al., 2003; Simoncini et al., 2004). In a previous study (Vos et al., 2002) with radiolabeled tibolone, 17β-monos metabolites constituted less than 10% of the radiolabel administered. We have also determined the 3α- and 3β-di-S metabolites because their levels were very high in the pilot study, and these di-S metabolites were considered as end-product for sulfation.

Gas chromatography with mass spectrometry procedures have been validated for human serum, and liquid chromatography/tandem mass spectrometry procedures have been validated for human serum, myometrium, and breast tissue with regard to selectivity, sensitivity, calibration curves, accuracy, precision, stability, dilution, and carryover. The procedures have been used for monkey plasma and tissues without further validation because no monkey control tissue was available for validation. Detection limits were 0.1 to 0.5 ng/ml (plasma) and 0.5 to 2 ng/g (tissues). Analytes in monkey plasma and tissues were determined with acceptable precision (coefficient of variation <20% for overall, within batch, and between batch) and accuracy (bias...
<20%), except for the mono-S metabolites. Quality control samples for the mono-S metabolites showed that the bias was <20% for low and medium concentrations, resulting in a maximal 60% overestimation. Because mono-S metabolites contributed little to the metabolite patterns, potential overestimation at lower levels was accepted.

For metabolites with concentrations outside the calibration range, a "best estimate" of the concentration is given, provided that the peak exceeded the background by at least 3-fold; if lower, a best estimate of "0" was assigned.

Calculations. The area under the concentration-time curve from 0 to 24 h (AUC0–24), maximum plasma concentration (Cmax), and time to maximum plasma concentration (Tmax) were calculated using WinNonlin version 4.1 on SAS version 9.1.2 (SAS Institute, Cary, NC). Missing values were estimated by interpolation from adjacent time points. Plasma and tissue ratios and percentages of metabolites within tissues have been calculated using AUC.

Results

Plasma. Cmax, Tmax, and AUC0–24 of tibolone and metabolites at necropsy are presented in Table 1, and plasma concentrations versus time plots are shown in Fig. 2 (A, nonsulfated metabolites; B, sulfated metabolites). Tibolone was not detectable. The predominant nonsulfated metabolite was the estrogenic 3αOH-tib; the AUC of the other estrogenic metabolite, 3βOH-tib, was about 20-fold lower. The progesterogenic androgenic metabolite, Δ4-tib, had about an 8-fold lower AUC than 3αOH-tib. The predominant sulfated metabolite was 3αS,17βOH-tib; considerable levels of 3βS,17βS-tib were present; and levels of mono-S metabolites were low. Peak levels of the free and di-S metabolites were reached after 1 and 1.25 h, respectively. Plasma concentrations of all the metabolites decreased with time (Fig. 2, A and B). The plasma pharmacokinetic parameters of tibolone metabolites at necropsy, based on one monkey per time point, are comparable with the pharmacokinetic results obtained at day 36, based on six monkeys per time point (Verheul et al., 2007), e.g., the AUC at day 36 were 0.1, 69, 5, 14, 127, 3922, 43, and 1801 for tibolone, 3αOH-tib, 3βOH-tib, Δ4-tib, 3αS,17βOH-tib, 3αS,17βS-tib, 3βS,17βOH-tib, and 3βS,17βS-tib, respectively. The concentration-time curves at necropsy and at day 36 are superimposable. This indicates that the plasma results at necropsy obtained in a single monkey per time point are a good reflection of the results obtained in six animals.

Tissues. The AUC of the metabolites in the heart muscle were <3% of those in plasma, indicating that the tissues had been adequately washed free of blood. Tibolone levels were undetectable in tissues and are not presented in tables and figures.

Efficacy Tissues. Brain. Tibolone metabolite patterns were qualitatively comparable in all the brain tissues measured, but quantitative, regional differences were found as shown by the AUC (Table 2). The hypothalamic concentration-time curve is shown in Fig. 3A as a representative brain tissue. The predominant free metabolite in brain tissues was the estrogenic 3αOH-tib, with peak levels at 1 to 2.25 h and decreasing to 10 to 20% at 24 h (Fig. 3A). The predominant sulfated metabolite in most brain regions was 3αS,17βS-tib, with peak levels later and lower (3–20 times) than the corresponding 3αOH-tib. AUC of 3βS,17βS-tib were lower than those of 3αOH-tib (Table 2), and AUC of mono-S metabolites were relatively high. Levels of all the sulfated metabolites decreased with time and were less than 1 ng/g at 24 h (data not shown). Assuming that plasma levels (ng/ml) and tissue levels (ng/g) may be compared, AUC in most brain areas were higher for 3αOH-tib, 3βOH-tib, and Δ4-tib than in plasma. In contrast, AUC of di-S metabolites in most brain tissues were considerably lower, ranging from 1/25 times in midbrain to <1/100 times in other brain regions.

Vagina. The predominant free metabolite in distal vagina was 3αOH-tib. In proximal vagina, the androgenic/progesterogenic Δ4-tib metabolite had the largest AUC of the three nonsulfated metabolites (Table 2), although the difference with 3αOH-tib is not large. In both distal and proximal vaginal tissues, 3αS,17βS-tib was the predominant inactive sulfated metabolite, followed by 3βS,17βS-tib (Table 2). The Tmax were 4 and 1 to 2.5 h for di-S metabolites and for mono-S and free metabolites, respectively. AUC of mono-S metabolites were considerably (6 times) higher in proximal vagina. Compared with plasma, AUC in vagina tissues were lower, except for 3βOH-tib and Δ4-tib and for mono-S metabolites (proximal vagina only). Metabolite patterns in vaginal tissues are different from the metabolite patterns in brain and plasma.

Safety Tissues. Uterus. The AUC of the estrogenic metabolite, 3αOH-tib, in endometrium was 8-fold higher than that of 3βOH-tib (Table 3; Fig. 3B). After their peak at 1 h, both decreased rapidly. In contrast, Δ4-tib levels initially increased for about 4 h and became higher than those of 3αOH-tib and 3βOH-tib (Fig. 3B). The predominant sulfated metabolite in endometrium was 3αS,17βS-tib, whereas the mono-S metabolites in endometrium were 40- to 50-fold lower than those of the di-S. The metabolite pattern in myometrium was comparable with the endometrial pattern. Compared with plasma, AUC of free and sulfated metabolites were lower in uterine tissues, except those of Δ4-tib, which were considerably (8- to 10-fold) higher. Compared with brain and vagina, the AUC of di-S and Δ4-tib in uterus were higher. The metabolite pattern in uterus is different from those in plasma, brain, and vagina.

Breast. The predominant metabolites in glandular breast tissue were di-S metabolites (Table 3) with peak levels at 4 h. AUC of 3αOH-tib and 3βOH-tib were 30 times and >100 times lower (Table 3). The AUC of Δ4-tib was comparable with that of 3αOH-tib. As in the
endometrium, levels of 3αOH-tib ($T_{\text{max}} = 1$ h) in the breast rapidly decreased, whereas those of $\Delta^4$-tib ($T_{\text{max}} = 2.25$ h) appeared to decrease later in time. Compared with fat breast tissue, AUC of mono-S and di-S metabolites in glandular tissue were higher (1.5-fold), whereas those of free metabolites were lower (0.5-fold). Compared with plasma, AUC in glandular and fat tissue of S-metabolites were lower (3-fold); those of 3αOH-tib and 3βOH-tib were comparable; and those of $\Delta^4$-tib were higher. Compared with vaginal tissue, in particular proximal vagina, the AUC of the mono-S in breast tissues were lower. In both breast tissues and uterus, the AUC of the di-S metabolites were large, but the AUC of the $\Delta^4$-tib were lower in mammary glands and higher in mammary fat tissues. The metabolite pattern in breast tissues is different from those in the efficacy tissues, brain, and vagina and from that in uterus.
**Ratios.** We calculated various ratios and percentages to characterize and compare the qualitative metabolite profiles independent of the actual levels and to illustrate differences in metabolite patterns in different tissues (Tables 2 and 3). In brain tissues, percentages of free, nonsulfated metabolites were high, in contrast to plasma and all the nonbrain tissues. Percentages of mono-S metabolites, which can be back-converted to active estrogenic metabolites, were less than 10%, except in most brain tissues and proximal vagina. This indicates that sulfated metabolites in plasma, distal vagina, uterus, and breast predominantly (>95%) occur in the di-S form. The difference in percentage of mono-S/total-S between proximal (39%) and distal (9%) vagina was remarkable. In all the tissues and plasma, 3αOH-tib was predominant over 3βOH-tib, with ratios ranging from >20 in brain to about 2 in vagina. The ratio [(tib + Δ²-tib)/ (3αOH-tib + 3βOH-tib)] favored the estrogenic (3αOH-tib + 3βOH-tib) metabolites in plasma and all the brain tissues, was about equal in the vagina (proximal and distal), breast (glands and fat), and myometrium, and was clearly toward the progestagenic and androgenic (tibolone + Δ²-tib) metabolites in endometrium. These ratios illustrate that tibolone has unique metabolite patterns in plasma and tissues.

**Discussion**

This report presents levels of tibolone and its free and sulfated metabolites in plasma and, for the first time, in tissues. The summary in Fig. 4 shows that tissues have unique tibolone metabolite patterns, which, in turn, are different from that in plasma.

As in humans, the predominant nonsulfated metabolite in monkeys is 3αOH-tib, whereas levels of tibolone and Δ²-tib are low and rapidly decrease over time. A quantitative difference from humans is the ratio of 3αOH-tib/3βOH-tib in plasma (2- to 35-fold versus a constant 3-fold in humans). This may be attributed to differences in species, formulation, or design (e.g., dose). The 0.5-mg/kg/day dose was used to enhance the chance to detect metabolites in plasma and tissues and also at longer intervals. Based on body weight, this is 10- to 15-fold, and on calorie intake, 2- to 3-fold higher than the human dose (Clarkson et al., 2002; Chine et al., 2002).

Enzymes involved in tibolone’s metabolism were described previously. Tibolone is rapidly hydrolyzed by AKR1C isoenzymes (Steckelbroeck et al., 2006). The liver-specific AKR1C4 preferentially forms 3αOH-tib, accounting for the predominance of 3αOH metabolites in plasma. AKR1C1 and AKR1C2 preferentially forming 3βOH-tib were shown in tissues resulting in 3βOH-tib predominance in tissues in vitro. However, our data show that 3αOH-tib is predominant in monkey tissues, suggesting that in vitro data should be extrapolated with caution to in vivo. The high degree of sulfation in plasma may be explained by the SULT2A1 enzyme in the stomach, liver, and intestine (Wang et al., 2006), which is able to convert tibolone and its metabolites to monosulfates and disulfates (Falany et al., 2004). Sulfation renders compounds inactive at receptors. Sulfa-

**TABLE 3**

<table>
<thead>
<tr>
<th>Plasma/Tissue</th>
<th>AUC</th>
<th>Percentage of 3OH Metabolites</th>
<th>Tissue Ratio Δ²/tib/ (3α OH + 3β OH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>58</td>
<td>3</td>
<td>151</td>
</tr>
<tr>
<td>Endometrium</td>
<td>22</td>
<td>3</td>
<td>66</td>
</tr>
<tr>
<td>Myometrium</td>
<td>38</td>
<td>5</td>
<td>57</td>
</tr>
<tr>
<td>Mammary glands</td>
<td>31</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td>Mammary fat</td>
<td>88</td>
<td>4</td>
<td>101</td>
</tr>
</tbody>
</table>

**FIG. 4.** AUC of tibolone metabolites in hypothalamus, proximal vagina, endometrium, mammary glands, and plasma. The combined AUC are presented of the estrogenic metabolites [sum of (3αOH-tib + 3βOH-tib)], the progestagenic/androgenic [sum of (Δ²-tib + tib)] metabolites, the mono-S [sum of (3αS,17βOH-tib + 3βS,17βOH-tib)] metabolites, and the di-S [sum of (3αS,17βS-bis(tib + 3βS,17βS-tib))].

Sulfation renders compounds inactive at receptors. Sulfation at the 17-position seems to be irreversible (de Gooyer et al., 2001) as shown in rats with double-labeled estradiol sulfate (Takanashi et al., 2003). Two studies suggest, however, that 17-sulfation may, to some degree, be reversible (Goldzieher et al., 1988; Simoncini et al., 2004).

Both AUC and concentration-time curves of tibolone metabolites in plasma at necropsy from one monkey per time point were similar to AUC and concentration-time curves obtained on day 36, based on the mean of six monkeys. Thus, it would appear that—despite single measurements at necropsy—these results are a good reflection of the actual situation. The time-dependent concentration-time curves in tissues also support this conclusion.

Our results show that tibolone metabolites have unique patterns in various tissues. Brain tissues contain high levels of 3-hydroxymetabolites and low levels of di-S metabolites and a considerable percentage of mono-S metabolites (Fig. 4). One explanation may be that only more lipophilic, nonsulfated metabolites can pass the blood-brain barrier. However, this would not explain the higher levels of free metabolites in brain compared with plasma. Recently, organic anion transporter proteins have been found in various tissues, including the brain (Kullak-Ublick et al., 1998; Steckelbroeck et al., 2004). These proteins can transport 3-sulfated steroids, such as estrone sulfate and dehydroepiandrosterone sulfate, across membranes. Assuming that these organic anion transporter proteins described in human temporal lobe are expressed in monkey brain and that they act on other 3-sulfated steroids, it would explain the relatively high levels of the 3-mono-S tibolone metabolites in brain tissues. Mono-S metabolites entering the brain can then readily be desulfated by sulfatases (Gold-
leading to receptor-active 3αOH-tib and 3βOH-tib. Thus, the 3-mono-S metabolites can serve as a reservoir for estrogenic activity. These two mechanisms, transporters for 3-mono-S steroids and local presence of sulfataes, can explain high levels of free and mono-S metabolites in brain tissues. The regional differences in brain mono-S and di-S metabolite levels may be explained by a site-specific balance between SULT and sulfatase activity; for example, the lack of activity and mRNA expression of SULT in the temporal lobe (cortex) of human brain (Kullak-Ublick et al., 1998) may account for the low di-S levels in the cortex, whereas different SULT activities as shown in different brain tissues in rats (Aldred and Waring, 1999) may explain regional differences in sulfation. The region-specific expression of SULT4A1 in human brain further supports this notion (Liyou et al., 2003), when SULT4A1 can also sulfate tibolone and its metabolites. Irrespective of these mechanisms, our data show that estrogenic 3αOH-tib and 3βOH-tib are predominant in brain, including the hypothalamus, which is primarily involved in control of hot flushes (Stearn et al., 2002). This estrogenic metabolite pattern is in line with the observed estrogenic effects on the brain, i.e., a reduction in hot flushes as shown in ovariectomized monkeys (Jelinek et al., 1984) and postmenopausal women (Landgren et al., 2005). However, estrogenic effects of tibolone on the pituitary seem not to be very strong because tibolone only partly reduces follicle-stimulating hormone levels in postmenopausal women (Doeren et al., 2001) and cynomolgus monkeys (Gibbs et al., 2002), in contrast to continuous combined regimens resulting in near-complete suppression. Unfortunately, the pituitary was too small for assessment of hormone levels.

Compared with the brain, the proximal vagina has a different metabolite pattern (Fig. 4) with higher progestagen/estrogen ratios. The vaginal balance is only slightly toward estrogenic metabolites, but this is apparently sufficient for favorable vaginal effects of tibolone (Kloosterboer, 2001). Tibolone on vaginal atrophy and dryness observed in postmenopausal women is too small for assessment of hormone levels.

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**References**


Address correspondence to: H. A. M. Verheul, NV Organon, KR6419, Molenstraat 110, 5320BH Oss, The Netherlands. E-mail: herman.verheul@organon.com