

Title page

Pharmacokinetic parameters of tibolone and metabolites in plasma, urine, feces and bile from ovariectomized cynomolgus monkeys after a single dose or multiple doses of tibolone.

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Abbreviations

Aldo keto reductase (AKR); coefficient of variation (CV%); di-sulfate (di-S); estrone (E₁); estrone sulfate (E₁S); estradiol (E₂); Gaschromatography followed by mass spectrometry (GC-MS); liquid chromatography with tandem mass spectrometry (LC-MS/MS); liquid-liquid extraction (LLE); Lower level of Quantitation (LloQ); multiple dose (md); single dose (sd); mono-S (mono-sulfate); pharmacokinetic (PK); solid phase extraction (SPE); Selective Tissue Estrogen Activity Regulator (STEAR); Sulfotransferase (SULT); tibolone (tib).

Abstract

Levels of non-sulfated and sulfated tibolone metabolites were determined in plasma, urine, and feces from 6 ovariectomized, mature female cynomolgus monkeys after a single dose and multiple oral doses (including bile) of tibolone using validated GC-MS and LC-MS/MS assays. In plasma, the predominant non-sulfated metabolite after single and multiple dosing was the estrogenic 3 α -hydroxytibolone; levels of the estrogenic 3 β -hydroxytibolone were 10-fold and of progestagenic/androgenic Δ^4 -tibolone 5-fold lower. Tibolone was undetectable. The predominant sulfated metabolite was 3 α S,17 β S-tibolone; levels of 3 β S,17 β S-tibolone were about 2-fold and -mono-sulfated 3-hydroxymetabolites about 10-fold lower. After multiple doses, AUCs of non-sulfated metabolites were lower (2-fold), and of sulfated metabolites 25% higher. In plasma, > 95% metabolites were di-sulfated. In urine, levels of all metabolites after single and multiple doses were low. After a single dose, high levels of 3 β -hydroxytibolone and the 3-mono-sulfated metabolites [3 β S,17 β OH-tibolone and 3 α S,17 β OH-tibolone] were found in feces. After multiple dosing, 3 α -hydroxytibolone increased and the ratio of 3 α /3 β -hydroxytibolone became about 1. The predominant sulfated metabolite was 3 α S,17 β S-tibolone. Levels of all metabolites in feces were higher after multiple than after a single dose. Levels of non-sulfated and 3-mono-sulfated metabolites were higher in feces than in plasma. Bile contained very high metabolite levels, except mono-sulfates. This may contribute to the metabolite content of the feces after multiple doses. 3 β -Hydroxytibolone and 3 α S,17 β S-tibolone predominated. In conclusion, tibolone had different metabolite patterns in plasma, urine, feces and bile in monkeys. The bile contributed to the metabolite pattern in feces after multiple doses. Feces was the major excretion route.

In randomized clinical trials, tibolone (Livial[®], Org OD14; (7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one) has in early postmenopausal women estrogenic effects on vasomotor symptoms, vagina and bone. No or only transient estrogenic effects on breast and endometrium (Kloosterboer, 2004; Rymer et al., 2005; Landgren et al., 2005) are found as indicated by its neutral effects on mammographic density, a low incidence of tenderness and no or only initial, transient vaginal bleeding. These tissue specific effects may result from metabolism to different active metabolites and from a balance between activation by sulfatase and deactivation by the sulfotransferases (see Figure 1). This defines tibolone as a selective tissue estrogenic activity regulator (STEAR) (Kloosterboer, 2004). Previously reported (Vos et al., 2002; Timmer and Huisman, 2002; Timmer et al., 2002; Timmer and Houwing, 2002; Timmer and Doorstam, 2002) pharmacokinetic (PK) data in postmenopausal women have been limited to tibolone and its active, non-sulfated metabolites [3 α -hydroxytibolone (3 α OH-tib), 3 β OH-tib and Δ^4 -tib]. The predominant metabolite in blood is 3 α OH-tib followed by 3 β OH-tib. Levels of tibolone and Δ^4 -tib after a single dose (sd) and after multiple doses (md) are low in postmenopausal women and become undetectable after 4-6 hours. The 3 α OH-tib and 3 β OH-tib bind to the estrogen receptors and tibolone and Δ^4 -tib to the androgen and progestagen receptors (Kloosterboer, 2004; Gooyer et al., 2003). In a PK analysis (Vos et al., 2002) using radiolabelled tibolone, qualitative metabolite patterns have been determined with HPLC in plasma, urine and feces from 3 healthy, postmenopausal subjects, indicating that over 75% of the metabolites in the circulation are mono- or di-sulfated and that over 80% of the radiolabel is excreted after 192h, predominantly in the feces. Whereas sulfation renders compounds inactive at receptors, the enzyme sulfatase may readily reconvert 3-mono-sulfated, but not 17-mono-sulfated metabolites into active receptor binding metabolites (Goldzieher et al., 1988; Gooyer et al., 2001; Takanashi et al., 2003; Simoncini et al., 2004).

As in postmenopausal women, pharmacological studies with tibolone in monkeys have shown that tibolone reduces hot flushes (Jelinek et al., 1984) and that 0.2 mg/kg/d tibolone for 2 years protects ovariectomized cynomolgus monkeys against bone loss, without stimulation of breast and endometrium (Clarkson et al., 2002, 2004; Cline et al., 2002; Williams et al., 2002). These results show a high similarity with the effects in postmenopausal women. In monkeys, only a very limited pharmacokinetic profile of tibolone has been determined: plasma levels of 3α OH-tib, 3β OH-tib and Δ^4 -tib, one hour after dosing with 0.2 mg/kg/d tibolone were shown to be comparable to those after a 2.5 mg/d in postmenopausal women (Timmer and Houwing, 2002; Clarkson et al., 2001). In order to determine the pharmacokinetic profile of non-sulfated and sulfated tibolone metabolites, a study was conducted in ovariectomized cynomolgus monkeys: a single dose (sd) of 0.5 mg/kg tibolone followed, after a washout of 7 days, by multiple doses (md) from day 8 to day 44. Blood, urine, and feces were collected for 7 days after the dose on day 1 and 36. At necropsy (day 44), tissues, blood and bile were collected after the final dose of tibolone for their kinetic profiles. Since the bile is an excretion route for steroids and may also contribute to the steroid metabolite content of the feces, the results for the tibolone metabolites in the bile are reported here. Tissue results at necropsy will be reported elsewhere (Verheul et al., 2007). After development of analytically-validated assays, blood, urine, feces and bile have been assayed for their content of non-sulfated metabolites, 3-mono-sulfated (3-mono-S) metabolites, and di-sulfated (di-S) metabolites. This paper presents the levels of tibolone, its sulfated and its non-sulfated metabolites in plasma, urine, bile (md only) and feces from ovariectomized monkeys after sd and md of 0.5 mg/kg tibolone per day.

Materials and Methods

Collection of samples. Six mature, healthy, female cynomolgus monkeys (weighing 2.0-2.8 kg) were ovariectomized. After at least one month post-ovariectomy, the monkeys received a single dose, and after a washout of 1 week, they received repeated daily oral doses of tibolone (Org OD14; 0.5 mg/kg/d) from days 8-44 via nasogastric gavage. Cynomolgus monkeys were selected in view of the high similarity to humans with respect to effects on target and safety tissues. A dose of 0.5 mg/kg/d was chosen for this study to enhance the chance to measure levels of tibolone's metabolites in plasma, urine, feces, bile and tissues at the later time points. On day 1 and 36 plasma was collected at 0.5, 1, 2, 4, 6, 12 and 24 hours after dosing into tubes containing K₃EDTA, centrifuged (10 minutes at 2 000 x g within 1 hour after collection) and stored below -20 °C until analysis. The volume required for the determination of the non-sulfated metabolites exceeded the available sample volume of a particular monkey at a particular timepoint. Since the means of the individual plasma levels for the sulfated metabolites were demonstrated to be comparable to those of pooled samples, plasma samples were pooled per time point and these pools were used for the analysis of the non-sulfated metabolites. Urine and feces were collected in daily pools per individual for 7 days after day 1 (sd) and during steady-state after day 36 (md), respectively. On day 44, at 1, 1.25, 2.25, 4, 6 and 24 hours after the final dose, one animal was necropsied, and the bile was collected. This design was used to establish plasma, bile and tissue concentration-time curves, rather than to determine the concentrations at one single time point in 6 animals. The study was conducted at Huntingdon Life Sciences, USA and UK and complied with the Animal Welfare act regulations and GLP standards.

Preparation of feces and bile: Feces were weighed and homogenized with internal standards in 70% ethanol during 5-15 minutes using an ultraturrax at room temperature in cold buffers

(0-4 °C). Bile was weighed and the analytes with internal standards were extracted in 70% ethanol during 5-15 minutes. The homogenates of feces and bile samples were divided into aliquots and stored at 0-4 °C for at least 24 hours prior to further sample processing. The aliquots were centrifuged; the supernatant was evaporated, redissolved in water and stored at -20°C until analyzed as described below.

Assays: Tibolone, related reference compounds and deuterated tibolone standards, D₅-tib, D₅-3αOH-tib and D₃-Δ⁴-tib were supplied by NV Organon (Oss, The Netherlands). Other compounds were obtained from Sigma Aldrich.

For the determination of tibolone, 3αOH-tib and 3βOH-tib, the analytes and internal standard (D₅-tib, D₃-Δ⁴-tib, D₅-3αOH-tib) were extracted from plasma, urine, or from the homogenates of bile or feces by solid phase extraction (SPE) using 100 mg, 3 ml Isolute C18 (EC) columns (Sopachem, Wageningen, The Netherlands) and eluted with acetonitrile. The analytes were quantified without derivatization (tibolone) and with derivatization (3αOH-tib and 3βOH-tib) using tri-sil reagent (Pierce, Etten-Leur, The Netherlands) under alkaline conditions at room temperature with gas chromatography with mass spectrometry (GC-MS; Agilent 6890/5973 GC-MSD) and operated in the positive mode (ABL, Assen, The Netherlands). The concentration of tibolone was corrected for conversion to Δ⁴-tib during the analytical procedure using the ratio D₅-tib:D₅-Δ⁴-tib as correction factor. All other tibolone metabolites were quantified (at Xendo, Groningen, The Netherlands) by high performance liquid chromatography (Perkin Elmer Instruments, München, Germany), with tandem mass spectrometry detection (LC-MS/MS)(API4000; Applied Biosystems, Nieuwekerk aan de IJssel, The Netherlands). For the analysis of Δ⁴-tib, samples were mixed with an equal volume of ammonium formate buffer (pH 5.0) and extracted by liquid-liquid extraction (LLE) using 5 mL ethyl acetate:hexane (50:50 (v/v%)). The resulting organic

phase was evaporated, redissolved in water:methanol (50:50 (v/v%)) and subjected to LC-MS/MS analysis using a Synergi MAX-RP 80A column (Phenomenex, Torrance, USA) under isocratic conditions (35% ammonium acetate (pH 3.0), 65% methanol) and positive ionisation (turbo ion spray).

The selection of the sulfated metabolites to be assessed in validated assays was based on a pilot study with plasma pools, urine, liver and myometrium using semi-quantitative and non-validated LL-MS assays (Organon, Schaijk, The Netherlands). The di-sulfated (di-S) metabolites [3α S,17 β S-tib and 3 β S,17 β S-tib] proved to be present in high levels in plasma, urine and tissues. Both 3-mono-S metabolites [3α S,17 β OH-tib, 3 β S,17 β OH-tib] and 17 β -mono-sulfated metabolites [3α OH,17 β S-tib, 3 β OH,17 β S-tib and 17 β S-tib] were evaluated. The 3-mono-S metabolites were readily detected. However, 17 β -mono-S metabolites could only be detected in plasma pools, but not in urine and tissues: the AUCs in plasma were 143 and 255 ng/mL*h after sd and 201 and 273 ng/mL*h after md for 3α OH,17 β S-tib and 3 β OH,17 β S-tib, respectively. Low (< 5 ng/mL) levels of 17 β S-tib were detected only at 0.5 and 1h. This indicates that the levels of 17 β -mono-S metabolites were low, presumably due to the fact that they are readily sulfated to di-S metabolites. In a previous study (Vos et al., 2002) with radiolabelled tibolone, 17 β -mono sulfated metabolites constituted less than 10% of the radiolabel administered. Based on these results, the limited sample volumes, and since 17-mono-S compounds cannot readily be desulfated (Gooyer et al., 2001, Takanashi et al., 2003, Goldzieher et al., 1988, Simoncini et al., 2004) to receptor-binding metabolites, it was decided to develop validated assays for the di-S metabolites and only for 3-mono-S metabolites. The results obtained with the semi-quantitative analysis proved to be similar to those obtained with the validated assays.

For the analysis of the 3-mono-S metabolites in the quantitative and validated assays at Xendo (Groningen, The Netherlands), samples were extracted with ethyl acetate:hexane (50:50 (v/v%)) as described for Δ^4 -tib. The resulting water phase was subjected to on line solid phase extraction (SPE) using a Prospect 2 system with a HySphere C18 HD cartridge (Spark Holland B.V., Emmen, The Netherlands), followed by LC-MS/MS analysis using a Synergi MAX-RP 80A column (Phenomenex, Torrance, USA) under isocratic conditions (100% ammonium acetate (pH 6.0)) and negative ionisation (turbo ion spray). The di-sulfate (di-S) metabolites were isolated from the samples by protein precipitation with 2 volumes of acetonitrile. The supernatant was evaporated, redissolved in 100 μ L ammonium acetate (pH 6.0):methanol (70:30 (v/v)) and analyzed by LC-MS/MS using a Zorbax SB Phenyl column (Agilent Technologies through Bester, Amstelveen, The Netherlands) with an ammonium acetate (pH 6.0)/methanol gradient and negative ionisation.

The GC-MS procedures have been validated for human serum, LC-MS/MS procedures for human serum, myometrium and breast tissue with regard to selectivity, sensitivity, calibration curves, accuracy, precision, stability, dilution and carry-over. Validation procedures were guided by Shah (2000) and the FDA guidance for industry (2001). The procedures have been used for monkey plasma, urine, feces and bile without further validation. Detection limits were 0.1-0.5 ng/mL (for plasma and urine) and 0.5-2 ng/g (for feces and bile). Analytes were determined with acceptable precision (CV <20% for overall, within batch and between batch variation) and accuracy (bias <20%), except for the mono-S metabolites. Quality control samples for the 3-mono-S metabolites showed that the bias was >20% for low and medium concentrations, resulting in a maximal 60% overestimation. Despite this overestimation, the levels of the mono-S were very low and the mono-S metabolites seem to contribute little to the metabolite patterns. Therefore, the 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: $AUC_{0-t_{last}}$, AUC_{0-24h} or $AUC_{0.5-24h}$, C_{max} and t_{max} were calculated using WinNonlin version 4.1 on SAS version 8.2. If a value is missing, a best estimate was made by intrapolation of the results at adjacent timepoints. Means per metabolite and per time point were calculated. Ratios and percentages of metabolites were calculated by matrix, sampling time and by AUC.

Results

Plasma

The predominant non-sulfated metabolite after sd and md was $3\alpha\text{OH-tib}$ followed by $\Delta^4\text{-tib}$ (5-fold) and $3\beta\text{OH-tib}$ (10-fold) lower levels; see Figure 2 left upper and lower panels for concentration-time curves and Table 1 for the PK parameters (AUC , C_{max} , t_{max} and $t_{1/2}$). The levels rapidly declined in time. Tibolone was undetectable. After md, AUCs of all non-sulfated metabolites were approximately 2-fold lower than after sd. At 20-60-fold higher levels than the non-sulfated $3\alpha\text{OH-tib}$, the predominant sulfated metabolite after sd and md was $3\alpha\text{S},17\beta\text{S-tib}$ followed by $3\beta\text{S},17\beta\text{S-tib}$ (2-fold) and the 3-mono-sulfates (10-fold) lower than the corresponding di-S metabolites (Figure 2, right upper and lower panels, Table 1). AUC and C_{max} of di-S metabolites after md were about 25% increased compared to sd, whereas the AUCs of the mono-S metabolites remained the same. The t_{max} for the non-sulfated tibolone metabolites were 0.5 hours after both sd and md; the t_{max} for the mono-S metabolites changed from 1 hour after sd to 0.5 hour after md, whereas the t_{max} for the di-S metabolites remained 2 hours. The plasma concentrations of all metabolites, except for $3\alpha\text{S},17\beta\text{S-tib}$, returned to baseline at 24 hours after sd and md, indicating that these metabolites did not accumulate. The levels of the di-S metabolites at 24 hours after sd were similar to those after md. Compared to sd, $t_{1/2}$ of $3\alpha\text{OH-tib}$ and $3\beta\text{OH-tib}$ after md were reduced, whereas the $t_{1/2}$ of the di-S metabolites were higher (Table 1). To further characterize the metabolite profile in plasma independent of the actual levels and to allow comparison with the profiles in urine, feces and bile, we examined various ratios and percentages. The progestagen/estrogen ratio [(tibolone + $\Delta^4\text{-tib}$) / ($3\alpha\text{OH-tib}$ + $3\beta\text{OH-tib}$)] ranged from 0.1 to 0.4 after sd and md indicating that the balance is towards the 3OH metabolites in plasma. The $3\alpha/3\beta$ ratio [$3\alpha\text{OH-tib}/3\beta\text{OH-tib}$] was >10 . The 3α and 3β

metabolites were predominantly present in their sulfated forms and percentages of sulfated compounds increased in time from 80% at 0.5 hours to 98% at 24 hours and from 96 to 99% for sd and md, respectively, which is in-line with the sulfated percentages calculated using the AUCs (Table 1). The percentage of 3-mono-S metabolites decreased both after sd (18% at 0.5 hours to 4% at 24 hours) and md (9% to 1%). Based on AUCs, tibolone metabolites were present as di-sulfates for over 95% and 98% after sd and md, respectively.

Urine

Table 2 shows that after sd and md, 3 β OH-tib tended to be the non-sulfated tibolone metabolite in urine with the highest levels. The levels of tibolone were undetectable. The predominant sulfated metabolite was 3 α S,17 β S-tib. Levels of mono-S metabolites were 10-20-fold lower. Metabolite levels in urine after sd became undetectable after 3-4 days. It should be realized that after md, levels of the metabolites should be comparable for each of the 24-hour collection periods, since monkeys received a new dose of tibolone each morning. Compared to total amount of metabolites excreted during 0-168 hours after sd, the levels of non-sulfated metabolites in 24-hour urine samples after md were 2-4 x higher and the levels of the sulfated metabolites similar. In view of these low levels and a urinary volume of 100-120 mL/day, urine seemed to contribute very little to the excretion of tibolone. The 3 α /3 β ratio in urine was 0.8, clearly different from the ratios found in plasma.

Feces

The predominant non-sulfated and sulfated metabolites after sd in feces were 3 β OH-tib and the mono-S metabolite, 3 β S,17 β OH-tib, respectively (Table 3). As indicated previously, the levels after md should be comparable for each of the 24-hour collection periods. After md, the levels of the non-sulfated metabolites, 3 α OH-tib and 3 β OH-tib, were equally high, and Δ^4 -tib and tibolone were still present in considerable amounts (Table 3). The predominant

sulfated metabolite after md was the di-S metabolite, $3\alpha\text{S},17\beta\text{S-tib}$, followed by $3\beta\text{S},17\beta\text{S-tib}$. The mono-S metabolites were 30-40% of all sulfated metabolites. During all 24-hour collection periods after sd, the levels of all non-sulfated and sulfated metabolites were lower than after md. The concentration-time curves of the combined estrogenic ($3\alpha\text{-OH-tib}$ and $3\beta\text{OH-tib}$), progestagenic ($\text{tib} + \Delta 4\text{-tib}$), mono-S and di-S metabolites are presented in Figure 3 (left panel, sd and right panel md) and show that the levels of all metabolites after sd declined to <10 ng/g after 96 hours. The levels of the non-sulfated 3OH metabolites [$3\alpha\text{OH-tib} + 3\beta\text{OH-tib}$] were relatively high compared to those of the mono-S and di-S metabolites (Figure 3, left panel). Figure 3 (right panel) shows that the pattern of the three 24-hour collection periods after md were comparable. It also shows that the amount of the non-sulfated metabolites after md during a 24 hour period are about equal to the total amount (0-168 hours) after sd, whereas that of the di-S metabolites were 3-4-fold higher and that of $3\beta\text{S},17\beta\text{OH-tib}$ 3-fold lower. Comparing the results in urine (Table 2) with those in feces (Table 3) and taking the average urine volume (100-120 mL) and feces weight (about 60 g) into account, it is clear that the major route of excretion for tibolone and its metabolites was via the feces. As in plasma, the progestagen/estrogen ratio after sd and md in feces was low (<0.4), whereas –in contrast- the ratio of $3\alpha\text{OH-tib}/3\beta\text{OH-tib}$ was about 1. The percentage of sulfated metabolites after md was comparable to sd, whereas the percentage of mono-S was about 2-fold lower after md. Compared to plasma, the percentage of sulfated metabolites was about 2-fold lower in feces whereas the percentage of mono-S metabolites was more than 10-fold higher.

Bile

Bile was collected at necropsy after multiple doses of tibolone with one animal per time point. The levels of all non-sulfated metabolites were high at 1 hour and declined to baseline

levels at 24 hours, whereas the levels of di-S metabolites increased about 2-fold from 1 to 1.25 hours and were at 24 hours above 20,000 ng/g and 2,500 ng/g for the 3 α S,17 β S-tib and 3 β S,17 β S-tib, respectively. Based on AUC, 3 β OH-tib and 3 α S,17 β S-tib were the predominant non-sulfated and sulfated metabolites, respectively (Table 4). The progestagen/estrogen ratio was very low (<0.05) and the ratio of 3 α OH-tib/3 β OH-tib ranged from 0.2-0.6. The percentage of the mono-S metabolites was about zero in the bile. Compared to plasma, the AUCs and C_{max} of all metabolites in bile were considerably higher except for the 3-mono-S metabolites.

Discussion

This report presents for the first time the kinetic profiles of tibolone, its non-sulfated and sulfated metabolites in plasma, urine and feces from cynomolgus monkeys after a single and multiple doses of tibolone. At necropsy, after multiple doses, the metabolite profile is also determined in bile.

As in humans (Timmer and Huisman, 2002; Timmer et al., 2002; Timmer and Houwing, 2002; Timmer and Doorstam, 2002), the predominant, non-sulfated metabolite in monkey plasma is the estrogenic $3\alpha\text{OH-tib}$ with a t_{max} of about 0.5 hour; the levels of tibolone and the progestagenic/androgenic $\Delta^4\text{-tib}$ rapidly decline in time. The lower or comparable levels and AUCs in monkey plasma after md and sd, suggest that no accumulation of tibolone and its metabolites occurs in plasma, except for the di-S metabolites which have higher C_{max} (20-30%) and AUCs (about 50%) after md. However, the levels of the di-S metabolites at 24 hours after dosing were comparable between sd and md, suggesting no accumulation. A clear difference with humans is the ratio of $3\alpha\text{OH-tib}/3\beta\text{OH-tib}$ in monkey plasma (>10x versus 3x in humans) (Kloosterboer, 2004; Timmer and Huisman, 2002; Timmer et al., 2002; Timmer and Houwing, 2002; Timmer and Doorstam, 2002). This may be attributed to species differences or the study design (e.g. dose or formulation).

The data in plasma indicate that the most important phase I metabolic reaction is the rapid reduction of the 3-keto group to $3\alpha\text{OH-tib}$. In vitro studies have shown that the AKR1C4 enzyme expressed in liver (Stecklebroek et al., 2004) predominantly catalyzes the formation of $3\alpha\text{OH-tib}$. Non-sulfated OH-groups are rapidly sulfated, the 3-mono-S appearing slightly faster in plasma than the 3,17 di-S metabolites both after sd and md. In vitro studies have shown that tibolone and its metabolites can readily be sulfated at the C3 position by SULT2A1 (all tibolone metabolites, with a high affinity for $3\alpha\text{OH-tib}$), SULT1E1 (all

tibolone metabolites, except Δ^4 -tib) and SULT2B1b (3α OH-tib and 3β OH-tib only) (Falany et al., 2004). SULT2A1, expressed in liver was shown to be able to produce 3,17 di-S metabolites (Falany et al., 2004), explaining the high levels of the di-S in the circulation. Compared to sd, levels of non-sulfated metabolites in plasma after md are lower and those of sulfated, in particular the di-S metabolites, higher. A reduced expression of the AKR1C family members to explain the lower levels of the non-sulfated metabolites is less likely since the consequent increase in levels of tibolone or Δ^4 -tib has not been found. A more efficient sulfation e.g. by induction of SULTs, explains the lower levels of the non-sulfated metabolites and the shift towards higher levels of the di-S metabolites. It does, however, not explain the extent of the increase; the reduction in the AUCs of the non-sulfated metabolites is -by far- exceeded by the increase in the AUCs of the sulfated metabolites. In addition, the induction of SULT has only been described for SULT1E1 in endometrial tissues by progestagens, including tibolone (Falany and Falany, 1996). Other explanations for the extent of the increase in the di-S metabolites after md may be the contribution of the entero-hepatic circulation, which is obviously not yet present after sd. Another explanation may be the slower elimination of the di-S metabolites which is supported by the relatively high elimination half-life for di-S metabolites found in plasma.

Tibolone metabolites may be excreted via urine and feces. After sd, the metabolite pattern in feces shows relatively high levels of non-sulfated 3-hydroxymetabolites, tibolone, Δ^4 -tib, and of the mono-S metabolites whereas levels of di-S metabolites are relatively low. This pattern is different from that in plasma. Since the contribution of the bile to the feces is expected to be low in the early phase after sd, the metabolic pattern after a single dose seems to reflect the metabolic capacity of the gastro-intestinal tract. This pattern could be explained as follows: part of the tibolone dose is not absorbed but chemically converted in the stomach to Δ^4 -tib,

explaining the high levels of Δ^4 -tib in the feces; part of tibolone is metabolized in the intestine to 3-hydroxymetabolites by the AKR1C family present in the intestine (Stecklebroeck et al., 2004; Penning et al., 2000). The non-sulfated hydroxygroups can then be sulfated by sulfotransferases such as SULT2A1, SULT1E1 or SULT2B1b (Falany et al., 2004). Recently, SULT2A1 was shown to be present in the small intestine of postmenopausal women (Wang et al., 2006). The initial sulfation capacity in the intestine seems to be low in view of the high mono-S and the low di-S metabolite levels after sd in feces. After md, the concentrations of all metabolites during a 24-hour period are higher than those during the 0-24 hour period after sd. However, assuming that the total, excreted levels after sd (period 0-168 hours) is equivalent to a 24-hour period after md, the metabolite levels in the feces after sd are similar to those after md for all non-sulfated metabolites and $3\alpha\text{S},17\beta\text{OH-tib}$. After md, however, the levels of the di-S metabolites [$3\alpha\text{S},17\beta\text{S-tib}$ and $3\beta\text{S},17\beta\text{S-tib}$] were about 3-fold higher and of the mono-S metabolite, $3\beta\text{S},17\beta\text{OH-tib}$, about 3-fold lower than after sd. The reduction in the mono-S level after md may be explained by a more efficient sulphation. In addition, the bile may significantly contribute to the metabolite pattern in the feces after md, in view of its high concentrations of tibolone metabolites and since the bile weight at a particular moment (0.7 g) only reflects a part of the daily production and of the volume emptied in the intestine. Compared to the feces, the levels of metabolites in urine are much lower. Taking into account the mean weight of the feces (about 60g) and the urine volume (100-120 mL), it is clear that the major route of excretion of tibolone is via the feces. In the bile a higher levels of $3\beta\text{OH-tib}$ than of $3\alpha\text{OH-tib}$ are found after md, which is opposite to the situation in plasma. This may indicate that the $3\beta\text{OH-tib}$ is more efficiently absorbed from plasma or excreted by the liver, thus explaining the plasma predominance of

the 3α OH-tib, and the bile predominance of 3β -OH-tib. However, local metabolism in liver and bile of tibolone to the 3β OH-tib cannot be excluded.

It is concluded that after sd and md, 3α OH-tib and 3α S,17 β S-tib are the predominant non-sulfated and sulfated metabolites in plasma, respectively. Different tibolone metabolite patterns are observed with high levels of di-S metabolites in plasma, bile and urine, especially after md, whereas feces contained high levels of non-sulfated and mono-S metabolites. The bile contributes to the metabolite pattern in the feces. The major route of excretion for tibolone metabolites is via the feces.

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Figure legends

Figure 1 Structures of tibolone metabolites

Figure 2: Levels in plasma pools after a single dose and multiple doses of tibolone

Concentration-time plots of non-sulfated (panel A and B) and sulfated (panels C and D)

tibolone metabolites after a single (panel A and C) and multiple (panel B and D) doses of

tibolone are shown. It should be noted that Y-axes have different scales. Plasma pools were

made by mixing equal volumes from individual samples.

Figure 3 Tibolone metabolites in feces

Concentration-time plots of the non-sulfated and sulfated tibolone metabolites after a single

(panel A) and multiple (panel B) doses of tibolone are presented. Per collection period are

presented the combined levels of non-sulfated progestagenic metabolites [sum (Σ) of

(tibolone + Δ^4 -tib)], non-sulfated estrogenic metabolites [sum (Σ) of (3 α OH-tib + 3 β OH-

tib)], mono-S metabolites [sum (Σ) of (3 α S,17 β OH-tib + 3 β S,17 β OH-tib)] and the di-S

metabolites [sum (Σ) of (3 α S,17 β S-tib + 3 β S,17 β S-tib)]. In addition, the mean of the tibolone

metabolites during the three 24-hour periods after multiple doses is compared with the

cumulative amount (0-168 hours) of tibolone metabolites after a single dose. Note: the Y-

axes have different scales.

Table 1 Pharmacokinetic parameters and percentages in monkey plasma pools after a single dose and multiple doses of tibolone

Parameter	tibolone	Percentage										
		3 α OH-tib	3 β OH-tib	Δ^4 -tib	3 α S,17 β O H	3 α S,17 β S	3 β S,17 β O H	3 β S,17 β S	sulfate d	mono-S		
Single dose												
AUC ₀₋₂₄	0.1	129.0	9.0	22.3	179 \pm 56	2562 \pm 119	7	31 \pm 20	3	1188 \pm 46	97	5
C _{max}	0.0	36.0	2.8	4.6	35 \pm 14	338 \pm 168	9 \pm 4	203 \pm 81	93	8		
T _{max} (hour)	2.0	0.5	0.5	0.5	1.0	2.0	1.0	2.0				
T _{1/2} (hour)	nc	26.5	17.2	8.0	47.6	34.8	17.1	11.2				
Multiple doses												
AUC ₀₋₂₄	0.1	69.0	5.2	13.7	127 \pm 94	3922 \pm 124	1	43 \pm 27	7	1801 \pm 74	99	3
AUC _{0.5-24}	0.0	59.8	4.4	12.3	118 \pm 83	3821 \pm 120	40 \pm 19	1750 \pm 73	99	3		

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Percentage

						0		3		
C _{max} (ng/mL)	0.2	37.1	3.1	5.5	14±25	436±278	5±8	237±99	96	8
T _{max} (hour)	0.5	0.5	0.5	0.5	0.5	2	0.5	2		
T _{1/2} (hour)	nc	16.2	4.7	9.6	20.0	43.4	16.9	15.0		

AUC: Area under the curve in ng/mL*h; C_{max}: maximal concentration; t_{max}: time at C_{max}; AUC₀₋₂₄ and AUC_{0-tlast} have been calculated after sd.

After md the AUC₀₋₂₄ and AUC_{0.5-24} have been presented; nc: not calculated. The non-sulfated metabolites have been measured in plasma pools

only; therefore no SD can be given. % sulfated: 100% x (AUCs of mono-S + di-S metabolites)/(sum of AUCs of all metabolites); % mono-S:

(100% x (AUCs of mono-S metabolites)/(sum of AUCs of mono-S +di-S metabolites)

Table 2 Concentrations (ng/mL) of tibolone and non-sulfated and sulfated metabolites and percentages in monkey urine samples

Sampling time (h)	Mean level (\pm SD) of metabolite measured (ng/mL)								Percentage	
	tibolone	3 α OH-tib	3 β OH-tib	Δ^4 -tib	3 α S,17 β OH-tib	3 α S,17 β S-tib	3 β S,17 β OH-tib	3 β S,17 β S-tib	sulfated	mono-S
Single dose										
predose	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	nc	nc
0-24	0.0	0.0	0.3 \pm 0.4	0.6 \pm 0.2	4.3 \pm 5.2	95.0 \pm 80.6	2.7 \pm 3.8	48.0 \pm 26.3	99.0	4.6
24-48	0.0	0.7 \pm 1.4	1.7 \pm 3.7	0.1 \pm 0.2	1.7 \pm 1.4	46.2 \pm 34.0	0.8 \pm 0.9	8.8 \pm 6.0	95.4	4.4
48-72	0.0	1.0 \pm 1.1	0.6 \pm 1.0	0.1 \pm 0.2	0.4 \pm 0.3	6.0 \pm 4.3	0.2 \pm 0.2	1.0 \pm 0.4	81.1	7.2
72-96	0.0	0.1 \pm 0.2	0.1 \pm 0.1	0.0	0.1 \pm 0.1	1.3 \pm 1.1	0.0	0.2 \pm 0.1	86.4	5.7
96-120	0.0	0.0	0.0	0.0	0.0	0.2 \pm 0.2	0.0	0.1 \pm 0.1	85.1	8.8
120-144	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	nc	nc
144-168	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	nc	nc
0-168	0.0 \pm 0.0	1.9 \pm 2.2	2.8 \pm 3.6	0.9 \pm 0.3	6.4 \pm 5.6	148.7 \pm 77.5	3.8 \pm 3.9	58.1 \pm 27.9	96.4 \pm 2.8	4.5 \pm 2.5
Multiple dose										
-24 – 0	0.0 \pm 0.1	3.7 \pm 7.2	6.7 \pm 14.3	2.2 \pm 2.3	2.5 \pm 3.0	97.7 \pm 66.5	4.7 \pm 11.0	42.8 \pm 27.4	91.3	4.9
0-24	0.2 \pm 0.5	2.4 \pm 4.0	2.0 \pm 2.5	2.0 \pm 1.6	2.1 \pm 1.9	130.5 \pm 57.1	1.4 \pm 1.6	59.4 \pm 27.9	95.9	1.8
144-168	0.2 \pm 0.3	5.2 \pm 10.6	6.3 \pm 13.4	5.5 \pm 5.8	2.3 \pm 2.4	81.5 \pm 77.6	3.1 \pm 5.6	48.1 \pm 53.8	87.0	4.0
Mean \pm SD	0.1 \pm 0.3	3.8 \pm 7.4	5.0 \pm 11.0	3.2 \pm 3.9	2.3 \pm 2.3	103.2 \pm 66.8	3.1 \pm 6.9	50.1 \pm 36.7	91.4 \pm 4.5	3.6 \pm 1.6

See legend table 1. The mean (\pm SD) urine volume is 101 \pm 6 mL and 123 \pm 11 mL after sd and md, respectively

Table 3 Concentrations of tibolone and non-sulfated and sulfated metabolites and percentages in monkey feces samples

Sampling time (h)	Mean level (\pm SD) of metabolite measured (ng/g)								Percentage	
	tibolone	3 α OH-tib	3 β OH-tib	Δ^4 -tib	3 α S,17 β OH-tib	3 α S,17 β S-tib	3 β S,17 β OH-tib	3 β S,17 β S-tib	sulfated	mono-S
Single dose										
predose	0	0.0 \pm 0.1	0.4 \pm 0.5	0.0	1 \pm 1	0.0	0.1 \pm 0.3	0.0	57.3	100.0
"0-24h	16 \pm 23	250 \pm 181	401 \pm 462	70 \pm 64	91 \pm 81	71 \pm 93	505 \pm 505	67 \pm 110	48.7	81.2
24-48	12 \pm 8	280 \pm 108	437 \pm 247	57 \pm 38	107 \pm 54	59 \pm 73	372 \pm 259	43 \pm 44	41.7	82.4
48-72	6 \pm 5	150 \pm 131	97 \pm 94	25 \pm 22	56 \pm 20	44 \pm 67	103 \pm 80	22 \pm 33	43.4	70.7
72-96	0	48 \pm 47	14 \pm 10	2 \pm 1	15 \pm 12	16 \pm 24	32 \pm 42	7 \pm 12	53.5	68.8
96-120	0	22 \pm 22	5 \pm 5	1 \pm 1	10 \pm 8	7 \pm 8	3 \pm 3	2 \pm 3	45.6	61.5
120-144	0	4 \pm 4	1 \pm 1	1 \pm 1	2 \pm 4	2 \pm 2	1 \pm 2	1 \pm 1	45.6	52.0
144-168	0	8 \pm 14	6 \pm 14	4 \pm 8	7 \pm 11	2 \pm 3	3 \pm 8	2 \pm 5	40.9	71.0
0-168h	33\pm14	762\pm103	960\pm226	126\pm30	293\pm48	201\pm106	1020\pm313	144\pm80	44\pm7	82\pm4
Multiple dose										
-24 – 0	33 \pm 28	664 \pm 238	707 \pm 381	94 \pm 121	236 \pm 318	427 \pm 495	248 \pm 338	284 \pm 345	43.5	40.5
0-24	38 \pm 21	719 \pm 395	654 \pm 324	118 \pm 49	415 \pm 302	823 \pm 875	377 \pm 306	476 \pm 493	56.7	37.9
144-168	63 \pm 39	880 \pm 485	716 \pm 400	167 \pm 126	273 \pm 146	695 \pm 491	252 \pm 235	370 \pm 263	45.5	33.0

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Sampling time (h)	Mean level (\pm SD) of metabolite measured (ng/g)								Percentage	
	tibolone	3 α OH-tib	3 β OH-tib	Δ^4 -tib	3 α S,17 β OH-tib	3 α S,17 β S-tib	3 β S,17 β OH-tib	3 β S,17 β S-tib	sulfated	mono-S
Mean\pmSD	44\pm30	752 \pm 371	690\pm342	126\pm103	308\pm263	648\pm630	292\pm285	377\pm365	42\pm20	37\pm21

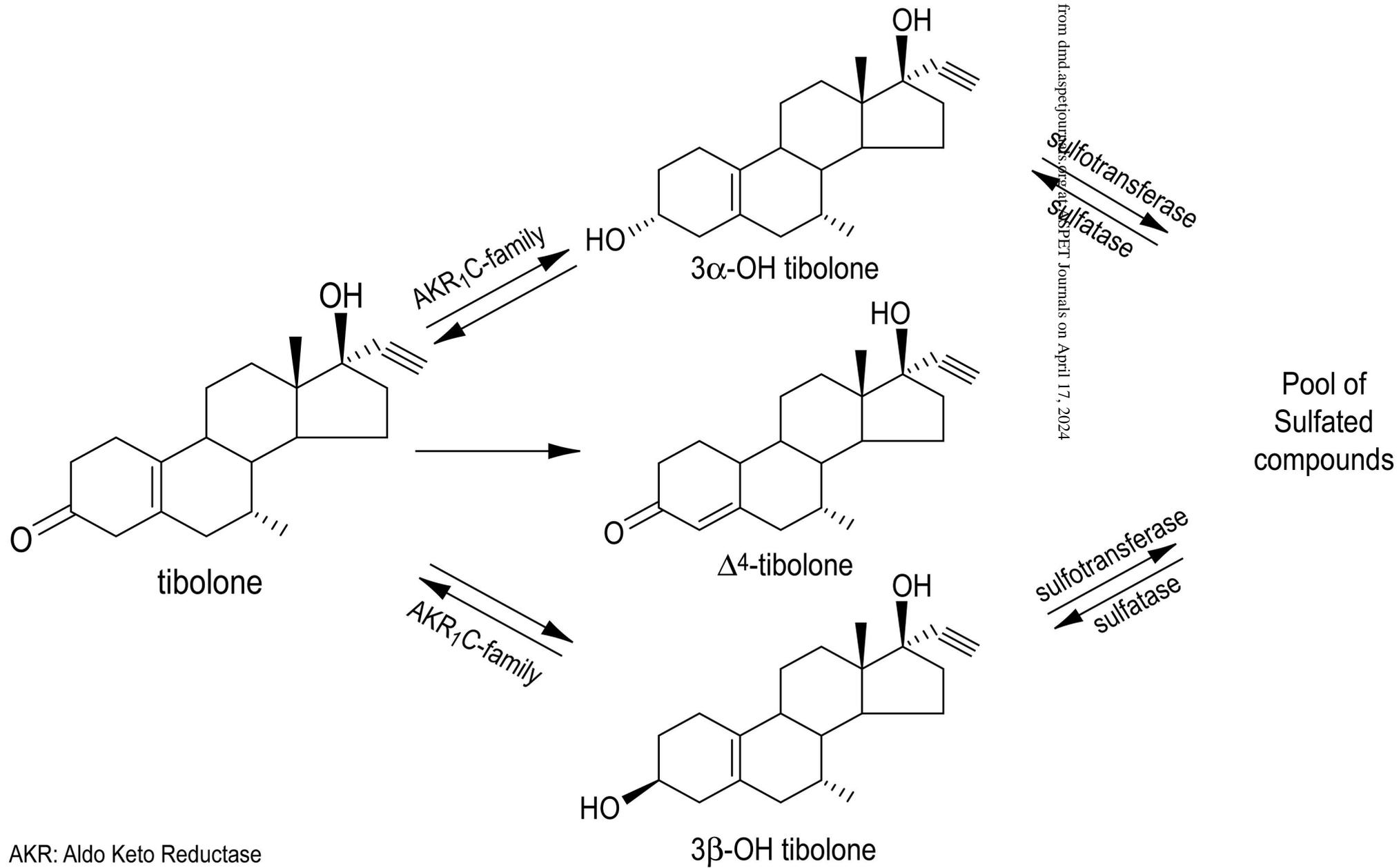
See legend table 1; The mean (\pm SD) weight of feces is 57 \pm 4 g and 61 \pm 4 g after sd and md, respectively.

Table 4 Pharmacokinetic parameters in monkey bile after multiple doses of tibolone

Parameter	tibolone	3 α OH-tib	3 β OH-tib	Δ^4 -tib	3 α S,17 β OH	3 α S,17 β S	3 β S,17 β OH	3 β S,17 β S	Percentage	
									sulfated	mono-S
AUC ₀₋₂₄	0	2280	4085	197	2	654828	42	254255	98	0
C _{max} (ng/mL)	0.0	364.0	970.0	59	3	54266	43	30447	98	0
T _{max} (hour)		1	1.25	1	1	1.25	1	1.25		

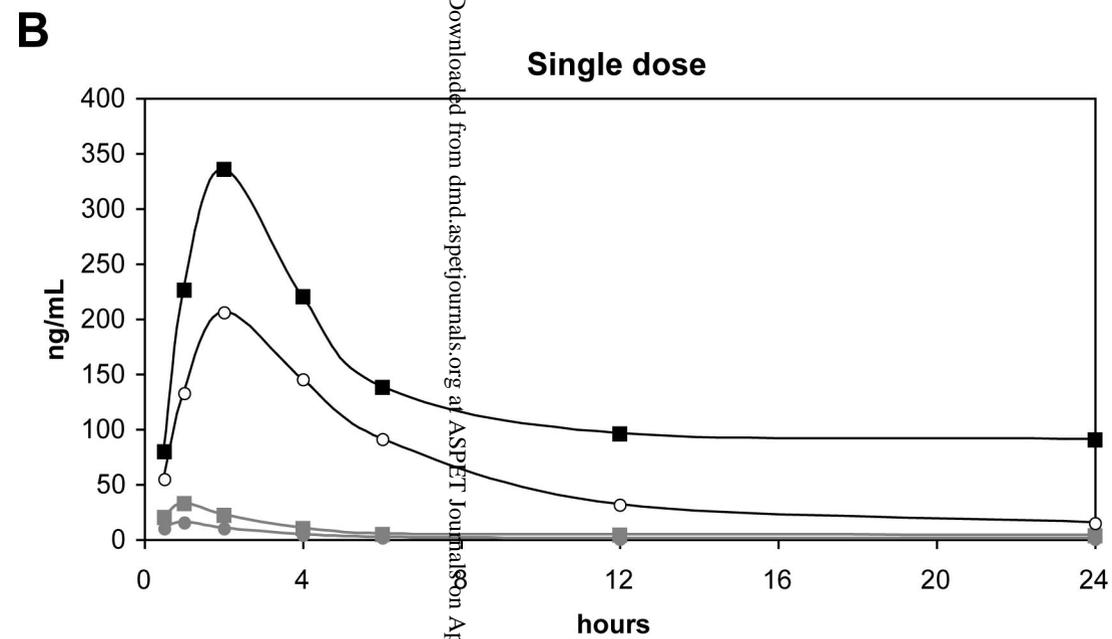
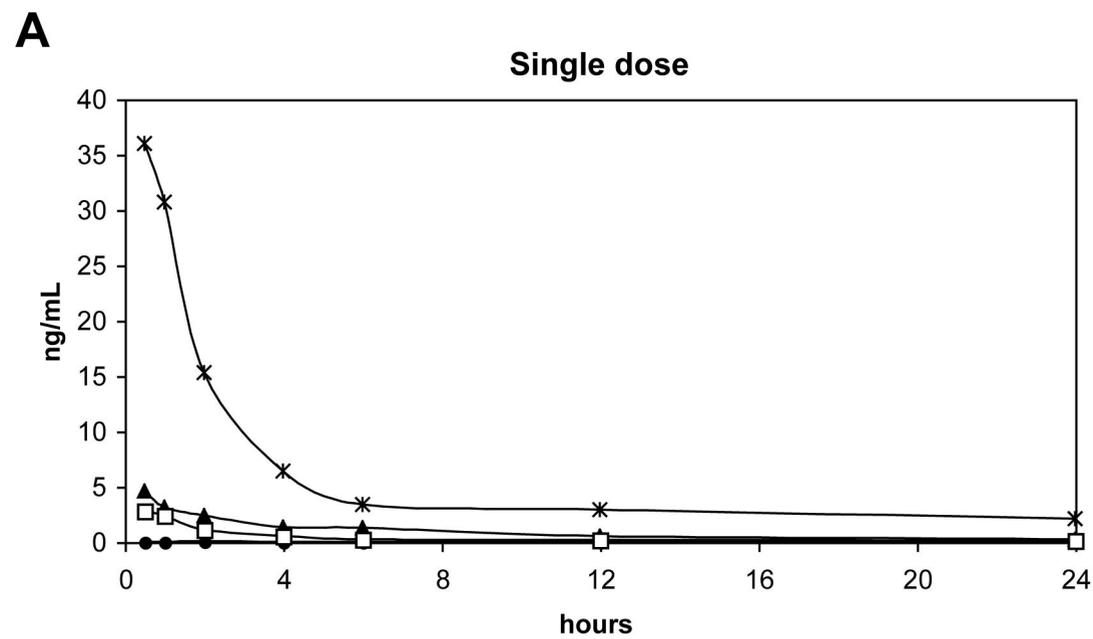
For legend see table 1; Note: data from the monkey necropsied at 6 hours after final dose have been excluded from the calculation for the PK parameters, since the total weight of the bile was very low. Mean (\pm SD) weight bile: 0.72 \pm 0.34 g

Figure 1

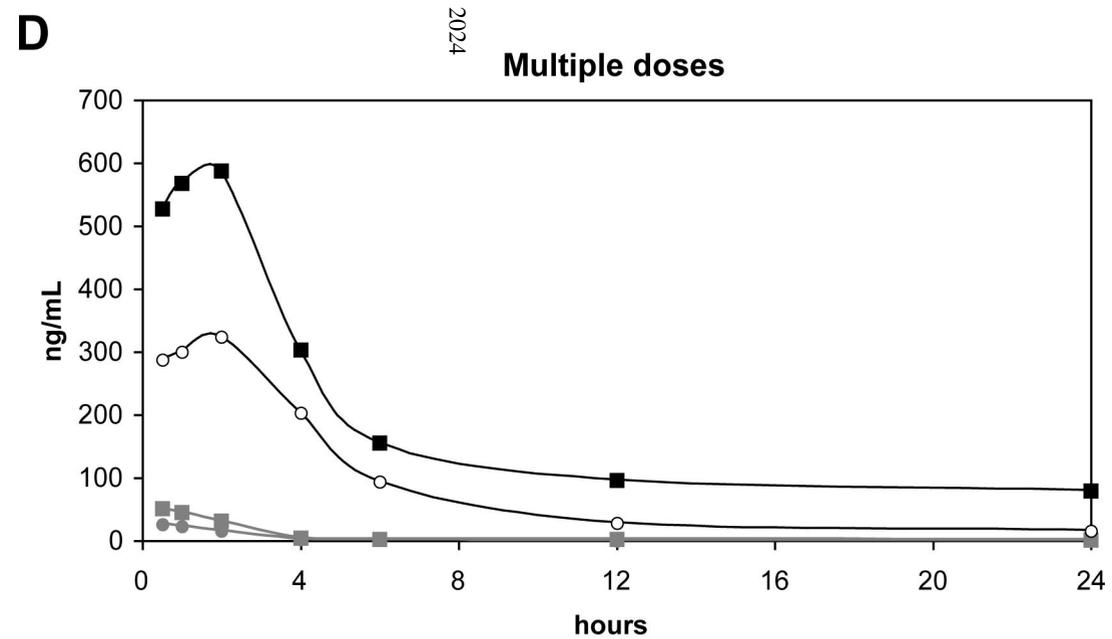
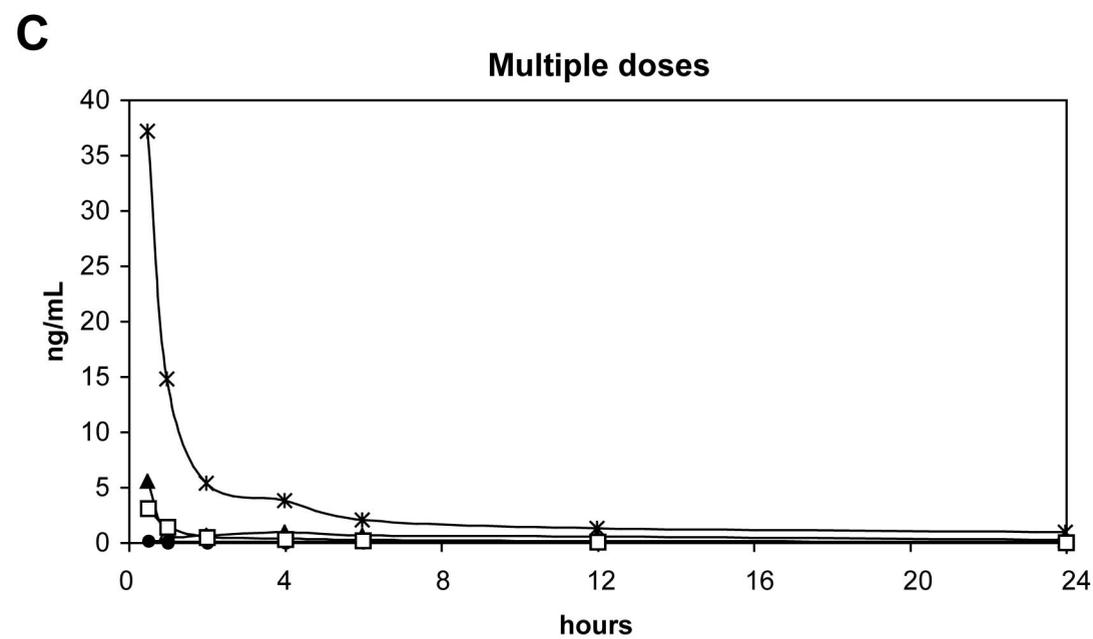


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Figure 2



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● tibolone ▲ Δ^4 -tib □ 3β OH-tib ✱ 3α OH-tib

■ 3α S,17 β OH-tib ■ 3α S,17 β S-tib ● 3β S,17 β OH-tib ○ 3β S,17 β S-tib

Figure 3

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