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Pharmacokinetics of three doses of sublingual testosterone in healthy premenopausal women

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Summary
Context: Sublingual testosterone is a single-dose treatment often used in studies regarding social, cognitive and sexual behavior. It is hypothesized that an increase in the ratio of free to total testosterone (free fraction) is indirectly, via genomic effects, responsible for the behavioral effects after sublingual testosterone administration.
Objective: To characterize the pharmacokinetics of three doses sublingual testosterone in premenopausal women. Also, to investigate the SHBG saturation threshold influencing the free level and free fraction of testosterone.
Design: We conducted an investigator-blind, randomized, cross-over placebo controlled study.
Setting: This study was undertaken at the research and development department of a scientific company for research regarding female sexual dysfunction.
Participants: 16 healthy premenopausal women (mean age 27.3 $\pm$ 5.3 years).
Interventions: Sublingual testosterone solution; 0.25, 0.50 and 0.75 mg.
Main outcomes measure: The pharmacokinetics of three single doses sublingual testosterone solution; the influence of SHBG levels on free and total levels of testosterone.
Results: After sublingual testosterone administration, serum free and total testosterone levels peaked at 15 min and reached baseline levels within 150 min. The AUCs and C_{max} of free and total testosterone differed significantly between the three doses ($p < 0.0001$) and increased dose-dependently.

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A dose-dependent increase in free fraction of testosterone was found in women with low SHBG levels, but not in women with high SHBG levels.
Conclusions: The three doses sublingual testosterone are rapidly absorbed and quickly metabolized in premenopausal women. These data demonstrate the influence of SHBG levels on the treatment induced alterations in plasma free testosterone.

1. Introduction

Results of scientific research indicate that testosterone is involved in social behavior (Bos et al., 2011; Eisenegger et al., 2011) including sexual behavior (Auger, 2004; Hull and Dominguez, 2007). Sexual behavior is influenced by endogenous testosterone levels as well as to exogenously administered testosterone. For exogenous testosterone administration, two different methods of treatment can be distinguished: chronic treatment vs single dose administration. Each method of treatment has its own pharmacokinetic profile, which may affect the influence of testosterone on behavior. Chronic testosterone administration is utilized as the delivery option in the majority of studies regarding the influence of testosterone on women's sexual behavior, including hormonal replacement therapy in naturally or surgically (bilateral oophorectomy) menopausal women (Shifren et al., 2000; Sherwin, 2002; Simon et al., 2005).

More recently however, several studies have investigated the effects of single dose testosterone administration on women's sexual behavior (Tuiten et al., 2000, 2002; van der Made et al., 2009). Tuiten et al. reported that a single sublingual dose of 0.50 mg testosterone significantly increased vaginal vasocongestion and experiences of sexual lust and genital sensation in premenopausal women without sexual complaints (Tuiten et al., 2000). These effects occurred 3—4 h after the induced testosterone peak and about 2 h after testosterone returned to baseline levels. This delay in behavioral effects after sublingual testosterone administration has been replicated in several other studies regarding social behavior and cognitive functions (Postma et al., 2000; Aleman et al., 2004; Schutter and van Honk, 2004; Hermans et al., 2006, 2007, 2008; Bos et al., 2010; Eisenegger et al., 2010; van Honk et al., 2001, 2004, 2005; van Honk and Schutter, 2007).

There are very few studies that have defined the pharmacokinetic profile of sublingual testosterone. Salehian et al. (1995), compared the pharmacokinetic profiles of 2 doses of sublingual testosterone (2.5 and 5.0 mg) with the pharmacokinetic profile of a long-acting testosterone ester, testosterone enanthate (TE) (in oil, im. 200 mg) in hypogonadal men. Compared to sublingual testosterone, the total and the free testosterone levels peaked days later in the male subjects studied who received TE. In the sublingual conditions the rise of free testosterone levels occurred within 1 h after administration, in the TE group this occurred 7 days after administration. Furthermore, it was shown that the free testosterone levels in the TE condition did not increase until the sex hormone binding globulin (SHBG) levels were suppressed after administration by day 7. The suppression of SHBG levels was significantly greater in the TE group than in either sublingual group (Salehian et al., 1995).

It is widely accepted that free testosterone is the biologically active testosterone (Mendel, 1989). Pharmacodynamic effects (measures of sexual functioning) would thus be expected to increase much later in the TE administered group compared to the sublingual administered group. Unfortunately, in the Salehian et al. study, post-dose sexual motivation was measured for the first time in the week before the first visit on day 20, when the free testosterone rise had already been passed in both groups. Notably, in the study by Tuiten and Van der Made et al., measures of sexual arousal increased 3 to 4 h after the peak of circulating testosterone (Tuiten et al., 2000; van der Made et al., 2009) and 2 h after testosterone levels returned to baseline (Tuiten et al., 2000), indicating that sublingual testosterone administration produces a pharmacodynamic effect after 4 h. Van der Made et al. suggested a SHBG saturation threshold hypothesis; i.e., when available binding sites of SHBG are occupied with testosterone after a sufficient single sublingual dose of testosterone, free fraction, and thus free testosterone levels increase thereby inducing behavioral effects (van der Made et al., 2009). The exact mechanism responsible for this delay in behavioral effect is not fully understood but it could be that testosterone exerts its behavioral effect via androgenic metabolites, genomic mechanisms (Bos et al., 2011) or a combination of these factors.

The main purpose of the present study was to establish an extensive pharmacokinetic profile of three different single doses of sublingual testosterone administered as a solution with cyclodextrin. The primary pharmacokinetic endpoints were levels of total and free testosterone. Secondary endpoints included the pharmacokinetics of 5α-dihydrotestosterone (DHT), and 3α-androstanediol glucuronide (3α-diol-G). Additionally serum albumin, 17β-estradiol (E_2) and SHBG were measured.

Moreover, we compared the data of the present study with those of the Tuiten et al.'s pharmacokinetic study (Tuiten et al., 2000) with regard to the effect of single dose sublingual testosterone on circulating free and total testosterone levels. Furthermore we sought to determine at which level serum testosterone occupies the available binding sites of SHBG and serum free testosterone increases, i.e., the postulated SHBG saturation threshold mechanism by van der Made et al. (2009).

2. Subjects and methods

2.1. Study subjects

Eligible women were between 21 and 40 years, premenopausal and had a body mass index (BMI) between 18 and 30 kg/m^2. Exclusion criteria included a history of a hormone-dependent malignancy, endocrine disease, neurological problems, psychiatric disorder, cardiovascular condition,

hypertension, abnormal liver or renal function. Women taking medications that interfere with metabolism of sex steroids or had used testosterone therapy within 6 months before study entry were excluded also.

Women were recruited and enrolled from referrals, newspaper advertisements, the Internet, and an internal database of our lab. To determine eligibility, participants were screened 2 weeks prior to study entry. In addition to an assessment of medical history, all subjects received a physical examination including a 12-lead electrocardiogram, standard biochemistry and hematological laboratory tests. Blood samples for determination of testosterone, SHBG, TSH, Thyroxine, FSH and estrogen were collected at baseline. A urine pregnancy test was applied to all women of child bearing potential.

16 healthy young women participated after providing written informed consent and received reimbursement for expenses for their participation. This study was approved by the local ethics committee (Stichting Therapeutische Evaluatie Geneesmiddelen Medisch Ethische Toetsingscommissie, Almere, The Netherlands) and carried out in agreement with ICH-GCP (International Conference on Harmonization – Good Clinical Practice).

2.2. Study design

This was a single-center, investigator-blind, randomized, cross-over placebo controlled study with three doses of a testosterone solution containing cyclodextrin administered sublingually. This solution consists of authentic nonmodified testosterone forming a soluble complex by a cyclodextrin carbohydrate ring. Due to increased solubility the absorption of testosterone through the oral mucosa is facilitated, thereby avoiding the hepatic first-pass metabolism (Brewster et al., 1988; Stuenkel et al., 1991; Salehian et al., 1995; Zhang et al., 2002).

All 16 subjects received each investigational drug dose once in random order. Wash-out between treatments was at least 48 h. Subjects had serial blood samples drawn via an intravenous catheter. Pharmacokinetic parameters were monitored at baseline and (at 2, 4, 6, 8, 10, 20, 30, 60, 90, 120, 180, 230 min) after dosing.

Measurement of total testosterone, free testosterone, and DHT were performed at each sampling time; E_2 at −5, 60 and 230 min; 3α-diol-G at −5, 60, 120, and 230 min; SHBG and albumin prior to dosing and at 230 min. Blood samples in the placebo condition were only measured at −5, 10, 60 and 230 min.

Vital signs were measured at regular intervals and an electrocardiogram was performed prior to dosing and at the end of the experimental day. For each experimental day, subjects were asked to attend the visit in fasting state and they received a strict diet (low fat, no caffeine) during the experimental day to minimize the influence of pharmacokinetic parameters. Drug, alcohol and pregnancy screens were performed prior to experimental sessions.

2.3. Medication and dosing

Testosterone and placebo were administered sublingually in 4 separate experimental phases with either a 0.25, 0.50, 0.75 mg dose and placebo as a solution using a micropipette (Gilson Pipetman P1000) from a 1 mg/mL solution. The 0.25 mg, 0.50 mg, and 0.75 mg testosterone were dosed from different volumes of the 1 mg/mL solution. For the placebo solution 0.50 mL was administered.

The different doses were prepared by an unblinded research associate and administered by blinded research associates. The blinded research associate administered the solution into the subjects mouth under the tongue, the subjects were instructed to keep the solution sublingually for 1 min while moving the tongue slightly to optimize absorption. After 1 min the blinded research associate instructed the subject to swallow the solution.

2.4. Hormone assays

The assay used for the determination of total testosterone, free testosterone (after ultrafiltration), and DHT was High Performance Liquid Chromatography with Mass Spectrometric detection (LC/MSMS) (API 4000, AB Sciex). The method was validated with a lower limit of quantification (LLOQ) of 0.02 ng/mL for testosterone and DHT, and 0.001 ng/mL for free testosterone. The LC/MSMS assay is a reliable method for analysis of free testosterone and overcomes the known limitations of direct immunoassays in measurement of testosterone values in the lower range (Miller et al., 2004; Labrie et al., 2006).

E_2 was analyzed by a chemiluminescence immunoassay (Siemens), the LLOQ was 0.25 pmol/L. 3α-diol-G was measured by ELISA (BioVendor), the LLOQ was 0.25 ng/mL. SHBG was measured by an electrochemiluminescent assay (ECLIA, Roche). Albumin was measured by Roche Bromocresol Green (BCG) analysis (Roche).

2.5. Statistical analysis

The pharmacokinetic parameters were analyzed using the WinNonlin software (version 5.1). Pharmacokinetic parameters including area under the curve, $t = 0$ till $t = 230$ min (AUC_{0-230}), maximum concentration ($C_{max)}$ and time to maximum concentration (t_{max}) were calculated based on actual and baseline corrected individual concentration time curves. AUCs were estimated using the linear trapezoidal rule. Individual pharmacokinetic parameters AUC_{0-230} and C_{max} and corresponding dose normalized parameters were log transformed and analyzed using a mixed maximum likelihood analysis (PROC MIXED in SAS, version 9.1) including subject as a random factor and drug as a fixed effect factor. Contrasts were made of the least square means to compare the different doses. T_{max} was analyzed using a Wilcoxon rank sum test. This was based on the planned times corresponding to the actual t_{max} to prevent bias in analysis results based on differences in sampling times.

The baseline levels of total and free testosterone, DHT, E_2, 3α-diol-G, SHBG and albumin were calculated by taking the mean of the placebo, 0.25, 0.50 and 0.75 mg predose levels.

Overall analysis of the free fraction (free testosterone levels divided by total testosterone levels at each time point) was analyzed in a 3 Drug (0.25 mg vs 0.50 mg vs 0.75 mg) × 6 Time (t = 4, 6, 8, 10, 20, 30 min) repeated measures ANOVA, with Drug and Time as within subjects factors.

In order to meet normality assumptions, baseline SHBG values were log-transformed and Pearson's correlation coefficients were calculated to further investigate relationships between SHBG levels, total testosterone, free testosterone and free fraction percentage of testosterone.

Subsequently, we divided the subjects into two subgroups, on the basis of their baseline SHBG levels (mean of placebo, 0.25, 0.50, 0.75 mg predose levels). This subdivision was based on a median split of the baseline SHBG levels. One group ($N = 8$) with low SHBG levels ($\leq$63 nmol/L) and the other group ($N = 8$) with relatively high SHBG levels (>63 nmol/L). Independent samples *t*-test were used to assess free testosterone levels with SHBG as grouping variable (low vs high SHBG) for each post-dose measurement.

The dependent variable free fraction was analyzed in a 3 Drug (0.25 mg vs 0.50 mg vs 0.75 mg) $\times$ 6 Time ($t = 4$, 6, 8, 10, 20, 30 min) $\times$ 2 Group (SHBG low vs SHBG high) repeated measures ANOVA, with Drug and Time as within subjects factor and Group as between subjects factor. To analyze the effects of the within subject factors within each group separately, paired-samples *t*-test were used for each SHBG group for each post-dose measurement between the three doses. For all ANOVAs sphericity was not violated. For all analyses a (two-sided) *p*-value less than 0.05 was considered statistically significant. SPSS 16.0 was used for all statistical analyses.

3. Results

The baseline characteristics and hormone levels of the 16 study participants are outlined in Table 1. One subject was excluded from the 0.50 mg analysis due to an incorrect administration procedure of the testosterone solution.

3.1. Primary pharmacokinetic endpoints

The pharmacokinetic parameters of total and free testosterone are summarized in Table 2.

3.1.1. Total testosterone

The three doses (0.25, 0.50, 0.75 mg) produced maximum levels of total testosterone of 3.79, 5.31 and 6.73 ng/mL, respectively, at means of 15.6, 15.1 and 14.3 min (Fig. 1).

The C_{max} of total testosterone was significantly different ($p < 0.0001$) among the three doses. We found no statistically significant differences in T_{max} of total testosterone between the three dosages. The AUCs of total testosterone were also statistically significant different among the three doses ($p < 0.0001$) and showed a dose-dependent increase. The calculated half-life of total testosterone showed a significant difference between the 0.50 mg and 0.75 mg dose ($p = 0.0125$).

3.1.2. Free testosterone

Peak levels for free testosterone during the three dosages were 0.021, 0.032 and 0.043 ng/mL at means of 15.6, 14.4 and 12.8 min respectively (Fig. 2). There was a statistically significant difference between the three doses with respect to C_{max} of free testosterone ($p < 0.0001$). There were no statistically significant differences for free testosterone T_{max} between the three dosages. Free testosterone AUCs were statistically significant different between the three doses and increased dose-dependently. The differences between the free testosterone AUCs of the 0.25 mg vs 0.50 mg and 0.25 mg vs 0.75 mg have *P* values <0.0001, while the difference between the 0.50 and 0.75 mg was significant at $p < 0.01$. There were no statistically significant differences between the three doses for the calculated half-life of free testosterone.

For all doses, baseline levels for total- and free testosterone were reached by 150 min.

3.1.3. Bioavailability

To determine the absolute percentage of the sublingual testosterone dose which is absorbed in the systemic circulation, the fraction of absorbed testosterone needs to be calculated from the formula used also for the AUC calculation after intravenous dosing. Since we did not have an intravenous standard, we took the 0.25 mg dosage as reference value. Thus the bioavailability of the 0.25 mg was set at 100%, and for 0.50 and 0.75 mg were calculated as 69% (or 0.34 mg), and 58% (or 0.43 mg), respectively. The bioavailability of sublingual testosterone administration decreases with increasing doses.

Table 1 Baseline and clinical characteristics of the participants.

Characteristic	Value ($N = 16$)
Age (years)	27.3 ± 5.3
Race (no. (%))	
Caucasian	11 (69)
Black	2 (13)
Asian	1 (6)
Other	2 (13)[a]
BMI (kg/m^2)	23.5 ± 3.4
Contraceptive (no. (%))	
Hormonal	11 (69)
Combined oral contraceptive pill	8 (50)
IUD (levonorgestrel)	2 (13)
Vaginal ring (progestin and estrogen)	1 (6)
Non-hormonal	1 (6)
None	4 (25.0)
Total testosterone (ng/mL)	0.2 ± 0.1
Free testosterone (pg/mL)	1.9 ± 0.7[b]
DHT (ng/mL)	0.1 ± 0.03
3α-diol-G (ng/mL)	2.0 ± 1.9
E_2 (pmol/L)	207 ± 147[c]
SHBG (nmol/L)	114 ± 120
Albumin (g/L)	44.7 ± 1.5

Plus–minus values are means ± SD. To convert total testosterone to nanomoles per liter, multiply by 3.467; to convert free testosterone to picomoles per liter, multiply by 3467. To convert total DHT to nanomoles per liter, multiply by 3.44. To convert 3α-diol-G to nanomoles per liter, multiply by 2.13.
All baseline levels are means of placebo, 0.25, 0.50, 0.75 mg predose levels.
[a] The percentages do not sum up to 100% due to rounding of the numbers.
[b] Only measured in 11 subjects; 5 subjects had values below the LLOQ.
[c] Only measured in 15 subjects; 1 subject had a value below the LLOQ.

Table 2 Baseline corrected pharmacokinetic parameters of total- and free testosterone following administration of 0.25—0.75 mg sublingual testosterone.

	Dose (mg)	$t_{1/2}$[c] (min)	T_{max}[c] (min)	Baseline corrected AUC_{0-230}[d] (ng min/mL)	C_{max}[d] (ng/mL)	MRT[c] (min)
Testosterone (ng/mL)[a]	0.25	49.8 ± 16.0	15.6 ± 5.4	194 (37.2)	3.79 (39.9)	57.7 ± 12.2
	0.50	49.7 ± 22.4	15.1 ± 5.5	266 (37.6)	5.31 (37.8)	55.6 ± 13.9
	0.75	58.5 ± 24.6	14.3 ± 5.3	337 (34.7)	6.73 (39.6)	59.5 ± 16.4
Free testosterone (ng/mL)[b]	0.25	42.3 ± 14.6	15.6 ± 5.1	0.95 (51.8)	0.021(39.7)	52.6 ± 11.6
	0.50	55.7 ± 27.5	14.4 ± 5.5	1.51 (40.2)	0.032(37.6)	57.1 ± 15.6
	0.75	51.1 ± 26.4	12.8 ± 6.3	1.87 (47.8)	0.043(45.7)	51.4 ± 14.5

To convert total testosterone to nanomoles per liter, multiply by 3.467; to convert free testosterone to picomoles per liter, multiply by 3467. MRT, mean residence time.
[a] Total testosterone normal range = 0.14—0.66 ng/mL (Davison et al., 2005).
[b] Free testosterone normal range = 0.00072—0.0036 ng/mL (Davison et al., 2005).
[c] Mean ± SD.
[d] Geometric mean (%CV).

3.1.4. Free fraction

Our analyses showed a statistically significant effect of drug dose on the free fraction of testosterone (i.e., the ratio of free to total testosterone) during the $t = 4$ through $t = 30$ min measurements ($p = 0.002$). We also found a statistically significant difference for the C_{max} during $t = 4$ through $t = 30$ min between the 0.25 mg and 0.50 mg ($p = 0.003$) and between 0.25 mg and 0.75 mg doses ($p = 0.010$), but not between the 0.50 and 0.75 mg dose ($p = 0.381$) (Fig. 3).

As stated above, we expected to find a relationship between circulating SHBG and the increases in the free levels and the free fraction of testosterone induced by the different dosages of sublingual testosterone. Moreover, our experimental manipulations produced no statistically significant changes in SHBG and albumin levels between and on test days (data not shown).

In our study population we found a large between-subject variation in circulating SHBG levels. Baseline SHBG levels (log transformed) were correlated with total testosterone levels ($t = 20$ min): $r = .732$, $p < 0.0002$; $r = .930$, $p < 0.001$ and $r = .894$, $p < 0.001$ for the 0.25 mg, 0.50 mg and 0.75 mg dose respectively. Baseline SHBG levels (log transformed) were inversely correlated with free testosterone levels ($t = 20$ min): $r = -.702$, $p < 0.003$; $r = -.849$, $p < 0.001$ and $r = -.798$, $p < 0.001$ for the 0.25 mg, 0.50 mg and 0.75 mg dose respectively. For the free fraction levels and SHBG levels, we observed stronger correlations; $r = -.947$, $p < 0.001$; $r = -.938$, $p < 0.001$ and $r = -.944$, $p < 0.001$ for the 0.25 mg, 0.50 mg and 0.75 mg dose respectively on $t = 20$.

Because of this large between-subject variation we subdivided the subjects in two group based on a median split of the baseline SHBG levels. The low SHBG group had a mean SHBG baseline level of 44 nmol/L (±11), while the high SHBG group had a mean level of 183 nmol/L (±141).

3.1.5. Total testosterone and SHBG

In subjects with low SHBG, the three doses produced maximum levels of total testosterone of 3.18, 3.93 and 4.73 ng/mL, respectively, at 20 min after dosing. In subjects with high SHBG, the maximum levels of total testosterone were 5.00, 7.08 and 9.04 ng/mL after administration of the three doses sublingual testosterone. Between groups, total testosterone levels were statistically different for $t = 10$ till $t = 30$ min in the 0.25 and 0.50 mg dose, and in the 0.75 mg dose 6 till 30 min after dosing.

3.1.6. Free testosterone and SHBG

In subjects with low SHBG, the three doses produced maximum levels of free testosterone of 0.026, 0.039 and 0.048 ng/mL, respectively, at 20 min after dosing. In subjects

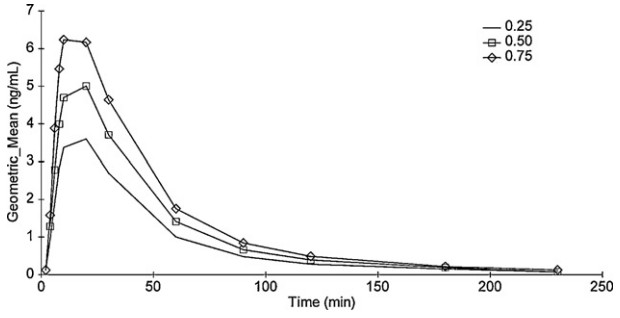

Figure 1 Geometric mean total testosterone levels in serum after administration of 0.25, 0.50 and 0.75 mg sublingual testosterone. Total testosterone normal range = 0.14—0.66 ng/mL (0.5—2.3 nmol/L) (Davison et al., 2005). To convert total testosterone to nanomoles per liter, multiply by 3.467.

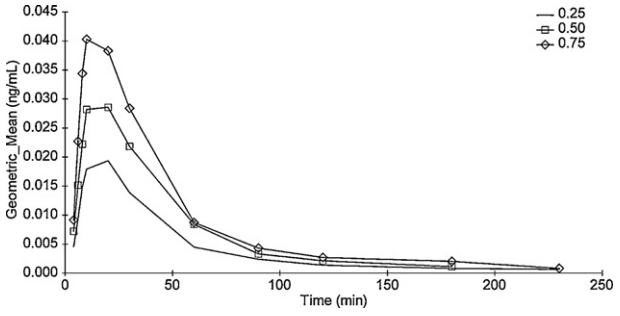

Figure 2 Geometric mean free testosterone levels in serum after administration of 0.25, 0.50 and 0.75 mg sublingual testosterone. Free testosterone normal range = 0.00072—0.0036 ng/mL (2.5—12.5 pmol/L) (Davison et al., 2005). To convert free testosterone to picomoles per liter, multiply by 3467.

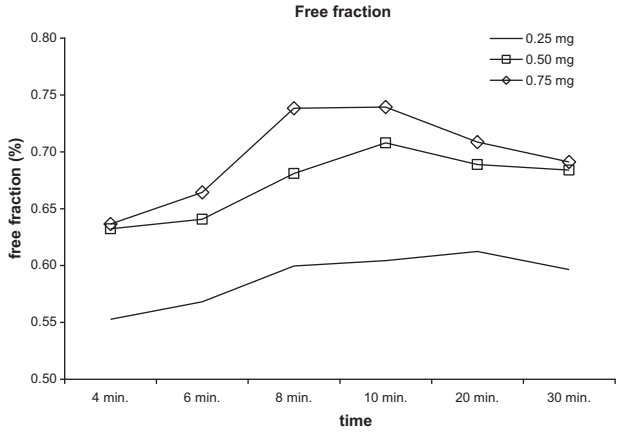

Figure 3 Free fraction of testosterone for 0.25 mg, 0.50 mg and 0.75 mg measured from $t = 4$ min to 30 min.

with high SHBG, the maximum levels of free testosterone were 0.018, 0.026 and 0.034 ng/mL after administration of the three doses sublingual testosterone. Between groups, all differences were statistically different, except for the levels of free testosterone in the 0.25 mg dose 4 and 20 min after dosing and in the 0.75 mg dose 4 and 10 min after dosing.

Our analyses showed that the low SHBG group had overall significantly higher levels of the free fraction compared to the high SHBG group ($p = 0.007$). Analyses revealed a statistically significant Group × Drug effect for the difference between 0.25 mg and 0.75 mg ($p = 0.012$) and between 0.25 mg and 0.50 mg ($p = 0.031$) (see Fig. 4). As shown in Fig. 4, statistically significant differences between the different doses sublingual testosterone were found in the low SHBG group.

3.2. Secondary pharmacokinetic endpoints

DHT peak levels of 0.285, 0.404 and 0.465 ng/mL were reached at means of 27.5, 28.0 and 27.5 min respectively (Table 3).

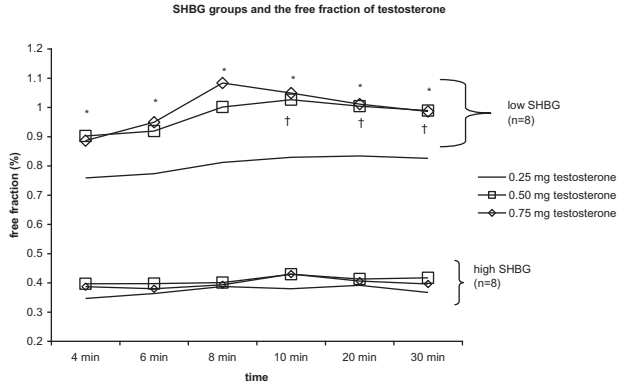

Figure 4 Free fraction of testosterone for 0.25 mg, 0.50 mg and 0.75 mg measured from $t = 4$ min to 30 min for the low and high SHBG groups. *Significant difference between 0.25 mg vs 0.75 mg ($p = <0.05$). †Significant difference between 0.25 mg vs 0.50 mg ($p = <0.05$).

T max differences between the three doses were not significant. The difference between the C_{max} of the 0.25 mg vs 0.50 mg and 0.25 mg vs 0.75 mg was significant ($p < 0.0001$), and the difference between the C_{max} of 0.50 mg and 0.75 mg was statistically significant ($p = 0.031$). Mean residence time of were not different the three sublingual doses. AUCs were statistically significant different between the three doses and increased dose-dependently.

The difference between the AUCs of the 0.25 mg vs 0.50 mg and 0.25 mg vs 0.75 mg was statistically significant ($p < 0.0001$), while the difference between the 0.50 and 0.75 mg was significant at $p = 0.021$. There were no statistically significant differences between the three doses, for the calculated half-life of DHT. For all doses, return to DHT baseline levels occurred within 180 min (Fig. 5).

Increasing doses of sublingual testosterone did not have a statistically significant influence on the 3α-diol-G concentrations as measured at $t = 0$, $t = 60$, $t = 120$, and $t = 230$. C_{max} and AUCs differences were not statistically significant between the three doses (data not shown).

E_2 levels did not change between the three doses of sublingual testosterone and did not increase significantly compared to baseline on $t = 60$ and $t = 230$ min (data not shown).

The three doses sublingual testosterone were well tolerated.

4. Discussion

Our results demonstrate that sublingual administration of each of the three doses testosterone was followed by a quick and steep increase of total and free testosterone levels; with peak levels reached at 15 min. Serum levels of total and free testosterone rapidly declined to reach baseline levels by 2.5 h, which is in line with our previous study (Tuiten et al., 2000), and with the reported pharmacokinetic profile following inhalation of testosterone (Davison et al., 2005).

The total testosterone C_{max} following administration of 0.50 mg sublingual testosterone showed consistency with the reported C_{max} of Tuiten et al. (2000). Also, the time to reach C_{max} of total testosterone in this study showed uniformity with the data of Tuiten et al. and the study of Salehian et al. (1995), who administered 2.5 mg and 5.0 mg sublingual testosterone.

DHT levels showed a significant dose-dependent increase, peak levels were reached within 30 min and levels returned to baseline levels within 3 h. DHT is metabolized to 3α-diol-G, so an elevation of 3α-diol-G levels was expected after administration of sublingual testosterone. However, no dose-dependent effect of sublingual testosterone on the concentration of 3α-diol-G was found.

According to the SHBG saturation threshold hypothesis by van der Made et al. (2009), an increased influx of testosterone into the body will occupy binding sites of SHBG. When most binding sites are occupied, free (non-SHBG bound) testosterone and consequently the free fraction will increase and thereby inducing, probably via genomic mechanisms (Bos et al., 2011), behavioral effects after approximately 4 h.

Table 3 Baseline corrected pharmacokinetic parameters of DHT following administration of 0.25—0.75 mg sublingual testosterone.

	Dose (mg)	$t_{1/2}$[a] (min)	T_{max}[a] (min)	AUC_{0-230}[b] (ng min/mL)	C_{max}[b] (ng/mL)	MRT[a] (min)
Dihydro-testosterone (ng/mL)	0.25	45.1 ± 10.5	27.5 ± 4.5	20.6 (44.9)	0.285 (42.5)	75.7 ± 14.4
	0.50	44.5 ± 16.8	28.0 ± 4.1	28.8 (37.9)	0.404 (37.6)	73.4 ± 14.8
	0.75	50.5 ± 30.4	27.5 ± 4.5	34.4 (41.3)	0.465 (43.5)	81.5 ± 36.3

DHT reference range = <0.29 ng/mL (Davison et al., 2005).
To convert total DHT to nanomoles per liter, multiply by 3.44.
[a] Mean ± SD.
[b] Geometric mean (%CV).

The results of the present study show that free and total testosterone levels significantly increase dose-dependently, which is reflected by an increase in the free fraction of testosterone. However, the difference in free fraction of testosterone between the 0.50 and 0.75 mg condition did not reach statistical significance. It is interesting that around T_{max} of free and total testosterone, six women have lower free fraction levels in the 0.75 mg condition compared to the 0.50 mg condition. Whether this is the result of variation in drug absorption, or the large between-subject variation in SHBG levels which could have influenced the results, is not clear. Furthermore, it is also possible that the number of subjects was probably too small to detect a significant increase in free fraction levels between these two doses.

Testosterone has a high affinity to SHBG and slowly dissociates from SHBG. Free testosterone is rapidly metabolized ($T_{1/2}$ 10 min.) which demonstrates the importance of SHBG binding and dissociation capacity, indicating that SHBG is the major determinant of the free fraction equilibrium. Fig. 4 shows the free fraction levels for subjects with low and high SHBG levels. In the low SHBG group we observed an increase of the free fraction of testosterone levels induced by increasing dosages of sublingual testosterone, while this pattern was not found in the women with high SHBG. These results corroborate the hypothesis of van der Made et al. (2009), namely: absorbed testosterone is bound to SHBG which has a limited capacity and only when this binding capacity is saturated, free testosterone and the free fraction increase.

According to van der Made, the increase in the free fraction might be responsible for behavioral effects observed 3.5—4 h later. However, in this study we measured free testosterone levels directly (with LC/MSMS) and we found these to be dose-dependently increased in both SHBG groups, in contrast to the free fraction which did not show a dose-dependent increase. Therefore we propose an adjustment to the SHBG saturation threshold hypothesis as postulated by van der Made et al. (2009); it is confirmed that SHBG levels influence the percentage of free fraction of testosterone (and the maximum concentration of free testosterone), however, an increase in free testosterone levels seems to be relatively less dependent of circulating SHBG levels after administration of the used dosages of sublingual testosterone. Further studies are necessary to investigate if free testosterone levels or free fraction levels are responsible to the observed behavioral effects as described by van der Made et al. (2009).

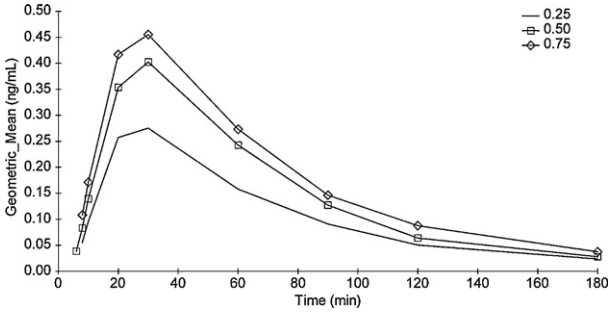

Figure 5 Geometric mean DHT levels in serum after administration of 0.25, 0.50 and 0.75 mg sublingual testosterone. DHT reference range = <0.29 ng/mL (Davison et al., 2005). To convert total DHT to nanomoles per liter, multiply by 3.44.

The data of the bioavailability show that sublingual testosterone absorption decreases with increasing doses and is 69% and 58% for the 0.50 and 0.75 dose respectively when the 0.25 mg condition is used as the reference value (100%). These data suggest a limitation of the total amount of testosterone absorbed. The volumes of the sublingual testosterone solution in the higher dose conditions were larger compared to the lower dosages. These increasing volumes could possibly influence the absorption at the limited surface area in the mouth.

In this study we did not take into account the cyclical and diurnal variation of testosterone. It is well known that testosterone levels are highest during the ovulatory and midluteal phase of the menstrual cycle and lowest in the early follicular phase and late luteal phase (Judd and Yen, 1973; Rothman et al., 2011; Salonia et al., 2008). In this study, blood samples were taken irrespective of menstrual cycle phase. However, almost 60% of the women in this study used some form of hormonal contraceptive (combined oral contraceptive pill, combined-contraceptive vaginal ring) which is known to suppress ovulation (Bancroft et al., 1991; Mulders and Dieben, 2001). Moreover, we assumed that the used dosages used in the present study overruled considerably the natural occurring relatively subtle cyclical and diurnal variation of testosterone. Furthermore, in a recent study by Braunstein et al. it was shown that SHBG levels of 161 women remained relatively stable across the menstrual cycle. They found a relatively small increase in testosterone levels in the mid-cycle period compared to the overall variability and suggest that the reference ranges described can be applied irrespective of the day in the menstrual cycle (Braunstein et al., 2011). So it is therefore unlikely that the dose-dependent increase in total and free

testosterone levels are biased by the cyclical and diurnal variation of testosterone.

Next to the sublingual route of testosterone administration other routes could be investigated as well. However for the desired immediate uptake and rapid return of testosterone to baseline levels the intramuscular and transdermal route are not suitable since both will result in gradual systemic uptake and prolonged higher plasma levels after drug administration via these routes. Oral administration is impossible at all, due to the very large first-pass effect no unmodified testosterone will reach the systemic circulation. For alternative routes next to sublingual with a very fast uptake and quick return to baseline of testosterone, the pulmonal and nasal delivery could be used for which in that case suitable and convenient dosage forms need to be developed.

In conclusion, the three doses testosterone are rapidly absorbed by the sublingual route and quickly metabolized without sustained elevations of DHT and E_2. These data suggest that a SHBG threshold exists which influences the increase in free fraction levels.

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Conflict of interest

Three authors (KvR, JB, HK) are employees of Emotional Brain (EB) and AT is CEO of Emotional Brain. KvR is researcher/physician at EB and PhD candidate. JB is researcher/psychologist at EB and PhD candidate. LdL is consultant to EB and Director at Exelion Bio-Pharmaceutical Consultancy BV. IG and EL declare that they have no conflict of interest. HK and BO are supervisors of the PhD theses of KvR. AT owns shares in EB.

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References

Aleman, A., Bronk, E., Kessels, R.P., Koppeschaar, H.P., van Honk, J., 2004. A single administration of testosterone improves visuospatial ability in young women. Psychoneuroendocrinology 29, 612—617.

Auger, A.P., 2004. Steroid receptor control of reproductive behavior. Horm. Behav. 45, 168—172.

Bancroft, J., Sherwin, B.B., Alexander, G.M., Davidson, D.W., Walker, A., 1991. Oral contraceptives, androgens, and the sexuality of young women: II. The role of androgens. Arch. Sex Behav. 20, 121—135.

Bos, P.A., Terburg, D., van Honk, J., 2010. Testosterone decreases trust in socially naive humans. Proc. Natl. Acad. Sci. U.S.A. 107, 9991—9995.

Bos, P.A., Panksepp, J., Bluthe, R.M., Honk, J.V., 2011. Acute effects of steroid hormones and neuropeptides on human social-emotional behavior: A review of single administration studies. Front. Neuroendocrinol., doi:10.1016/j.yfrne.2011.01.002.

Braunstein, G.D., Reitz, R.E., Buch, A., Schnell, D., Caulfield, M.P., 2011. Testosterone reference ranges in normally cycling healthy premenopausal women. J. Sex Med., doi:10.1111/j.1743-6109.2011.02380.x.

Brewster, M.E., Estes, K.S., Loftsson, T., Perchalski, R., Derendorf, H., Mullersman, G., Bodor, N., 1988. Improved delivery through biological membranes. XXXL: Solubilization and stabilization of an estradiol chemical delivery system by modified beta-cyclodextrins. J. Pharm. Sci. 77, 981—985.

Davison, S., Thipphawong, J., Blanchard, J., Liu, K., Morishige, R., Gonda, I., Okikawa, J., Adams, J., Evans, A., Otulana, B., Davis, S., 2005. Pharmacokinetics and acute safety of inhaled testosterone in postmenopausal women. J. Clin. Pharmacol. 45, 177—184.

Eisenegger, C., Haushofer, J., Fehr, E., 2011. The role of testosterone in social interaction. Trends Cogn. Sci. 15, 263—271.

Eisenegger, C., Naef, M., Snozzi, R., Heinrichs, M., Fehr, E., 2010. Prejudice and truth about the effect of testosterone on human bargaining behaviour. Nature 463, 356—359.

Hermans, E.J., Putman, P., van Honk, J., 2006. Testosterone administration reduces empathetic behavior: a facial mimicry study. Psychoneuroendocrinology 31, 859—866.

Hermans, E.J., Ramsey, N.F., van Honk, J., 2008. Exogenous testosterone enhances responsiveness to social threat in the neural circuitry of social aggression in humans. Biol. Psychiatry 63, 263—270.

Hermans, E.J., Putman, P., Baas, J.M., Gecks, N.M., Kenemans, J.L., van Honk, J., 2007. Exogenous testosterone attenuates the integrated central stress response in healthy young women. Psychoneuroendocrinology 32, 1052—1061.

Hull, E.M., Dominguez, J.M., 2007. Sexual behavior in male rodents. Horm. Behav. 52, 45—55.

Judd, H.L., Yen, S.S.C., 1973. Serum androstenedione and testosterone levels during menstrual-cycle. J. Clin. Endocrinol. Metab. 36, 475—481.

Labrie, F., Belanger, A., Belanger, P., Berube, R., Martel, C., Cusan, L., Gomez, J., Candas, B., Castiel, I., Chaussade, V., Deloche, C., Leclaire, J., 2006. Androgen glucuronides, instead of testosterone, as the new markers of androgenic activity in women. J. Steroid Biochem. Mol. Biol. 99, 182—188.

Mendel, C.M., 1989. The free hormone hypothesis: a physiologically based mathematical model. Endocr. Rev. 10, 232—274.

Miller, K.K., Rosner, W., Lee, H., Hier, J., Sesmilo, G., Schoenfeld, D., Neubauer, G., Klibanski, A., 2004. Measurement of free testosterone in normal women and women with androgen deficiency: comparison of methods. J. Clin. Endocrinol. Metab. 89, 525—533.

Mulders, T.M.T., Dieben, T.O.M., 2001. Use of the novel combined contraceptive vaginal ring NuvaRing for ovulation inhibition. Fertil. Steril. 75, 865—870.

Postma, A., Meyer, G., Tuiten, A., van Honk, J., Kessels, R.P., Thijssen, J., 2000. Effects of testosterone administration on selective aspects of object-location memory in healthy young women. Psychoneuroendocrinology 25, 563—575.

Rothman, M.S., Carlson, N.E., Xu, M., Wang, C., Swerdloff, R., Lee, P., Goh, V.H., Ridgway, E.C., Wierman, M.E., 2011. Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. Steroids 76, 177—182.

Salehian, B., Wang, C., Alexander, G., Davidson, T., McDonald, V., Berman, N., Dudley, R.E., Ziel, F., Swerdloff, R.S., 1995. Pharmacokinetics, bioefficacy, and safety of sublingual testosterone

cyclodextrin in hypogonadal men: comparison to testosterone enanthate: a clinical research center study. J. Clin. Endocrinol. Metab. 80, 3567—3575.

Salonia, A., Pontillo, M., Nappi, R.E., Zanni, G., Fabbri, F., Scavini, M., Daverio, R., Gallina, A., Rigatti, P., Bosi, E., Bonini, P.A., Montorsi, F., 2008. Menstrual cycle-related changes in circulating androgens in healthy women with self-reported normal sexual function. J. Sex Med. 5, 854—863.

Schutter, D.J., van Honk, J., 2004. Decoupling of midfrontal delta-beta oscillations after testosterone administration. Int. J. Psychophysiol. 53, 71—73.

Sherwin, B.B., 2002. Randomized clinical trials of combined estrogen-androgen preparations: effects on sexual functioning. Fertil. Steril. 77, S49—S54.

Shifren, J.L., Braunstein, G.D., Simon, J.A., Casson, P.R., Buster, J.E., Redmond, G.P., Burki, R.E., Ginsburg, E.S., Rosen, R.C., Leiblum, S.R., Caramelli, K.E., Mazer, N.A., Jones, K.P., Daugherty, C.A., 2000. Transdermal testosterone treatment in women with impaired sexual function after oophorectomy. N. Engl. J. Med. 343, 682—688.

Simon, J., Braunstein, G., Nachtigall, L., Utian, W., Katz, M., Miller, S., Waldbaum, A., Bouchard, C., Derzko, C., Buch, A., Rodenberg, C., Lucas, J., Davis, S., 2005. Testosterone patch increases sexual activity and desire in surgically menopausal women with hypoactive sexual desire disorder. J. Clin. Endocrinol. Metab. 90, 5226—5233.

Stuenkel, C.A., Dudley, R.E., Yen, S.S., 1991. Sublingual administration of testosterone-hydroxypropyl-beta-cyclodextrin inclusion complex simulates episodic androgen release in hypogonadal men. J. Clin. Endocrinol. Metab. 72, 1054—1059.

Tuiten, A., Van Honk, J., Koppeschaar, H., Bernaards, C., Thijssen, J., Verbaten, R., 2000. Time course of effects of testosterone administration on sexual arousal in women. Arch. Gen. Psychiatry 57, 149—153 discussion 155—156.

Tuiten, A., van Honk, J., Verbaten, R., Laan, E., Everaerd, W., Stam, H., 2002. Can sublingual testosterone increase subjective and physiological measures of laboratory-induced sexual arousal? Arch. Gen. Psychiatry 59, 465—466.

van der Made, F., Bloemers, J., Yassem, W.E., Kleiverda, G., Everaerd, W., van Ham, D., Olivier, B., Koppeschaar, H., Tuiten, A., 2009. The influence of testosterone combined with a PDE5-inhibitor on cognitive, affective, and physiological sexual functioning in women suffering from sexual dysfunction. J. Sex Med. 6, 777—790.

van Honk, J., Schutter, D.J., 2007. Testosterone reduces conscious detection of signals serving social correction: implications for antisocial behavior. Psychol. Sci. 18, 663—667.

van Honk, J., Peper, J.S., Schutter, D.J., 2005. Testosterone reduces unconscious fear but not consciously experienced anxiety: implications for the disorders of fear and anxiety. Biol. Psychiatry 58, 218—225.

van Honk, J., Schutter, D.J., Hermans, E.J., Putman, P., Tuiten, A., Koppeschaar, H., 2004. Testosterone shifts the balance between sensitivity for punishment and reward in healthy young women. Psychoneuroendocrinology 29, 937—943.

van Honk, J., Tuiten, A., Hermans, E., Putman, P., Koppeschaar, H., Thijssen, J., Verbaten, R., van Doornen, L., 2001. A single administration of testosterone induces cardiac accelerative responses to angry faces in healthy young women. Behav. Neurosci. 115, 238—242.

Zhang, H., Zhang, J., Streisand, J.B., 2002. Oral mucosal drug delivery: clinical pharmacokinetics and therapeutic applications. Clin. Pharmacokinet. 41, 661—680.