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## Measuring testosterone: the power of a method on steroids

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## salivary testosterone in female-to-male transgender adolescents during treatment with intramuscular injectable testosterone esters

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## ABSTRACT

In our hospital, female-to-male (FtM) transgender adolescents from the age of 16 are treated with two- or four-weekly intra-muscular injections of testosterone-esters. Some patients treated with four-weekly injections have complaints of fatigue and experience mood swings towards the end of the inter-injection period, which calls for an evaluation of the time-course of testosterone levels between injections. Evaluation of salivary testosterone is a practical approach for sequential measurements. Since only ~2% of total serum testosterone is present in saliva, a sensitive assay is necessary. The objective was to develop an isotope dilution-liquid chromatography-tandem mass spectrometry method (ID-LC-MS/MS) for salivary testosterone measurements and to evaluate the testosterone profiles after testosterone-ester mixture injections in FtM-adolescents.

**METHOD.** FtM treated with 125 mg/2 weeks or with 250 mg/4 weeks depots of testosterone-ester mixture collected saliva at different time intervals. Salivary testosterone was measured by a thoroughly validated ID-LC-MS/MS assay.

**RESULTS.** An ID-LC-MS/MS method for measuring salivary testosterone was developed with adequate accuracy and specificity. The reference range was established at 135-400 pmol/L. Testosterone levels peaked supra-physiologically immediately post-injection, and decreased to levels within the male reference range after nine days in all patients. 250 mg/4 weeks depots resulted in values below the reference range at the end of the 4 weeks.

**CONCLUSION.** The development of an adequate ID-LC-MS/MS method for measuring salivary testosterone allowed us to investigate the testosterone profile in FtM-adolescents after testosterone-esters mixture injections. These injections lead to extreme concentrations which may affect the wellbeing of the patients.

## INTRODUCTION

Transsexualism is the most extreme form of gender identity disorder (GID) and is accompanied by a strong wish for sex reassignment. At the Center of expertise on Genderdysphoria of the VU University Medical Center in Amsterdam, The Netherlands, adolescents who present with over-whelming signs of gender dysphoria enter a lengthy diagnostic process which ultimately may lead to treatment with cross-sex hormones from the age of 16 years [1]. Life long cross-sex hormone therapy is needed regardless of possible surgical removal of the gonads which can take place after the age of 18 years.

Cross-sex hormone treatment of female-to-male (FtM) transgender adolescents consists, in addition to GnRH analogs, of testosterone administration which, in our hospital, is administered as an intramuscular injection with a testosterone-esters

mixture (TEM). Testosterone is necessary to induce and maintain male secondary sex characteristics. The testosterone dose is increased every 6 months to a final dose of 125 mg every two weeks (induction) and subsequently to 250 mg every four weeks (maintenance) [1]. The latter is currently preferred because it minimizes the amount of intramuscular injections in an already very intensive process. However, some transgender adolescents complain about fluctuations in mood and energy in the week before the new injection, which might be caused by undesirably low testosterone levels during that week.

Serial testosterone measurements between injections could confirm whether this is indeed the case. In this setting, measurements in saliva are preferred above serum, as sample collection is non-invasive and hence can be performed without the intervention of a professional. Testosterone levels in saliva reflect serum free testosterone, since only this fraction of serum total testosterone is able to diffuse into saliva and saliva does not contain binding proteins [2;3]. However, as this fraction amounts to only 2-3%, salivary testosterone measurements require a highly sensitive assay. In this study, we develop a method to measure salivary testosterone concentrations with high sensitivity and accuracy and use this method to evaluate the profile of testosterone in saliva of FtM transgender adolescents after TEM injection.

## EXPERIMENTAL SECTION

### SUBJECTS

Eight adolescent female-to-male (FtM) transgender adolescents under treatment at the department of pediatrics at the VU University Medical Center and who were not suffering from gingivitis or mucosal bleeding gave informed consent to collect saliva between TEM injections: four FtM adolescents (age 17-19 years; BMI 20-27) were treated with 125 mg TEM per 2 weeks and four FtMs (age 18-22 years; BMI 20-30) were treated with 250 mg TEM per 4 weeks. Each patient was treated with the described TEM dose and frequency for at least 6 months before they entered this study. TEM injection consisted of Sustanon® 250 (Organon, Oss, the Netherlands) which is a mixture of several  $17\beta$ -hydroxyl esters of testosterone: 30 mg testosterone propionate, 60 mg phenylpropionate, 60 mg isohexanoate, and 100 mg decanoate, corresponding to 176 mg testosterone. Thirty self-proclaimed healthy male volunteers with Tanner stage G5 (16-60 years of age) donated saliva in order to determine reference values.

## SAMPLE COLLECTION

Patients received a time schedule and were instructed on how to collect saliva at home. Saliva collection did not take place within 30 min after brushing teeth. Study subjects were asked to rinse their mouth with tap water to remove potential food residue and to wait for at least 10 min. Moreover, during the collection period, study subjects were asked to refrain from using dental floss and eating food that could cause mucosal bleeding. Samples were collected by drooling at least 1 mL into a polypropylene tube without saliva flow stimulation. After saliva collection, the tubes were capped and stored in a regular house-hold freezer. The first sample collection took place directly before the testosterone esters injection. Other samples were collected 12, 24, and 72 h after injection and subsequently every two days until the next injection (2 or 4 weeks). After collection and storage at home, all samples of one patient were brought on ice to the clinic at the next appointment with the physician where the samples were stored at -20 °C until analysis.

## ANALYSIS AND QUANTIFICATION

*Instrumentation.* A Symbiosis online solid phase extraction (SPE) system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA) was used for the development of the salivary testosterone measurement. The Symbiosis system consists of a temperature controlled auto sampler, an automated cartridge exchange unit, two high pressure dispensers and a binary HPLC pump. Data acquisition and processing was done with Masslynx 4.1 software.

*Sample preparation.* All specimens were prepared in duplicate and a seven point calibration curve ranging from 13 to 3171 pmol/L was prepared with each batch. The testosterone stock standard used to prepare the calibration curve, has been calibrated by a serum total testosterone ID-LC-MS/MS method which was concordant with a published reference method, gas chromatography-mass spectrometry [4]. A stable isotopically labeled internal standard (testosterone-2,2,4,6,6-D<sub>5</sub>; D<sub>5</sub>T; obtained from CDN Isotopes, Canada) was dissolved in ethanol. With each batch, the internal standard was diluted with water (0.5 ng/mL) and 10 µL was added to 200 µL of every specimen (sample; control; calibrator) prior to work up. Testosterone was derivatized by adding 50 µL of aqueous 18% methoxylamine hydrochloride (MOX) (w/v) solution, vortex-mixing, and incubating at 80 °C for 1 h.

*Online SPE.* After derivatization, sample clean-up was performed by online SPE using a Hysphere C<sub>18</sub> cartridge. The cartridge was conditioned with methanol and equilibrated with 20:80 MeOH/H<sub>2</sub>O (v/v) containing 0.1% formic acid. Two hundred microliters of the derivatized testosterone (T-mox) solution was then loaded onto the cartridge with 20:80 MeOH/H<sub>2</sub>O (v/v) containing 0.1% formic acid (1 mL), and subsequently washed with 60:40 MeOH/H<sub>2</sub>O (v/v; 1 mL). Next, T-mox was eluted with

80:20 MeOH/H<sub>2</sub>O (v/v; 0.45 mL) from the cartridge directly to the LC column. The components were trapped on the analytical column by mixing 10:90 MeOH/ H<sub>2</sub>O (v/v) from the gradient pumps to the eluent. Finally, the cartridge was flushed with 0.5 mL H<sub>2</sub>O and put back into the tray for further use. Validation showed that cartridges can be reused up to six times without affecting the outcome of analysis.

*Liquid chromatography.* Separation was achieved on a C<sub>8</sub> analytical column (Waters, XBridge, 2.1 x 50 mm, 2.5 μm particle size), using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Gradient elution was executed according to the following program: 0-3 min, 90:10 A/B, 0.15 mL/min (focusing of SPE eluent); 3.20-4.30 min, 30:70 A/B (linear gradient) and kept there for 1.30 min; column flushed with 5:95 for 1 min; re-equilibration for 2 min at 95:5 A/B. The flow rate was 0.3 mL/min, except for the focusing step, and column temperature was kept at 40 °C.

*Tandem mass spectrometry.* Separated components were detected with MS/MS. Conditions were optimized for T-mox and ionization was carried out by electrospray in positive mode resulting in [M+H]<sup>+</sup> ions. For each component (analyte and internal standard), two transitions were monitored:  $m/z$  318 > 138 and  $m/z$  318 > 126 for T-mox;  $m/z$  323 > 142 and  $m/z$  323 > 129 for D<sub>5</sub>T-mox. The first transitions in each set were used for quantification, the second transitions for confirmation.

*Specificity.* Specificity was tested by spiking phosphate-buffered saline (0.9% NaCl, pH 7.4, B Braun, Melsungen, Germany) with supra-physiological amounts (72-100 nmol/L end concentrations) of epi-testosterone, dihydrotestosterone, androstenedione, 17-hydroxyprogesterone, estrone, estradiol, cortisol, dehydroepiandrosterone and dehydroepiandrosterone sulfate (Sigma-Aldrich, St Louis, MO, USA).

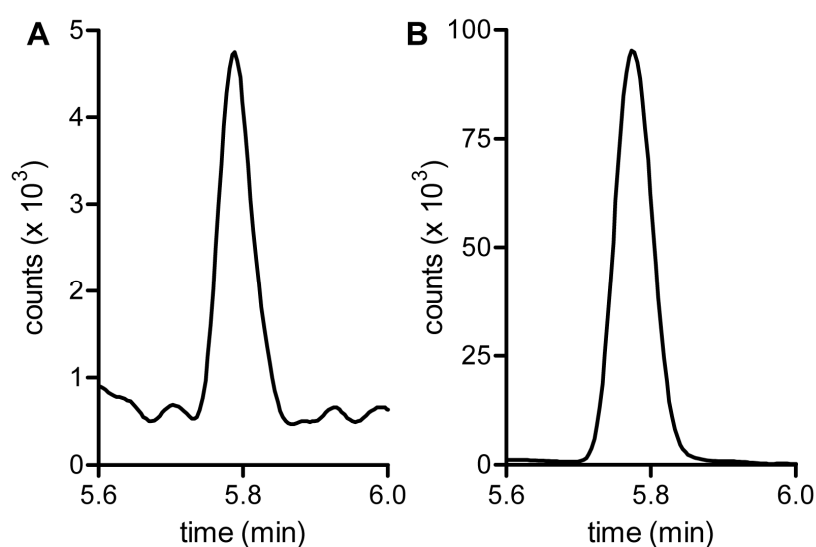
*Statistical analysis.* Statistical evaluation of the data was done using Medcalc 9.3 software (Medcalc Software, Mariakerke, Belgium). D'Agostino-Pearson test for normal distribution was used to determine the reference range. Patients groups were compared using Mann-Whitney test. P values ≤ 0.05 were considered to reflect statistical significance.

## RESULTS

### *Testosterone measurements in saliva: method validation*

The assay featured an inter-assay variation of 5% at 200 and 2000 pmol/L (n = 5). The intra-assay variation at 10, 140, and 900 pmol/L was 11%, 4%, and 2%, respectively (n = 12). Figure 1 displays the chromatograms of the quantifying transitions for testosterone and its internal standard, respectively, obtained by the analysis of a saliva

sample with 20 pmol/L testosterone. To determine a lower limit of quantification (LOQ), testosterone was measured in saliva, from a single female donor, in duplicate on 8 consecutive days. The sample measured a mean testosterone level of 8 pmol/L (equivalent to 0.3 pg on column) with a total error of 11%. Accuracy was tested by spiking 6 different saliva samples with three levels in two independent runs:  $n = 36$ ; recovery  $93 \pm 7\%$  (mean  $\pm$  SD). Specificity was demonstrated by spiking phosphate-buffered saline with supra-physiological amounts of different steroids. Androstenedione showed 0.4% interference, however this amount is not clinically relevant. All other steroids tested did not interfere with the measurement of testosterone.



**FIGURE 1:**

Chromatograms obtained by the analysis of a saliva sample containing 20 pmol/L testosterone. Left panel (A): quantifying trace for derivatized testosterone  $m/z$  318 > 138. Right panel (B): quantifying trace for the internal standard  $m/z$  323 > 142.

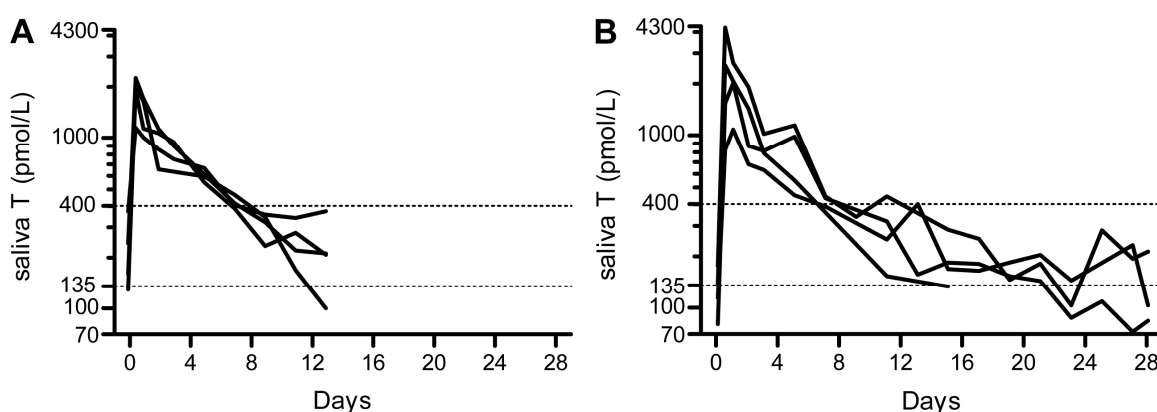
### Reference values

As the testosterone concentrations in healthy male subjects ( $n = 30$ ) were not normally distributed (D'Agostino-Pearson test ( $p = 0.0363$ )), the reference values were determined non-parametrically, which resulted in male reference values of 135 – 400 pmol/L.

### Salivary testosterone between TEM injections

The saliva collection in the first two days was complete for all subjects, whereas after day 2, 14 samples in total were not obtained. All eight FtM transgender adolescents showed a similar testosterone profile in saliva after TEM injection. The testosterone profiles of subjects receiving 125 mg TEM/2 weeks and 250 mg TEM/4 weeks, respectively, are presented in figure 2. In both groups, supra-physiological testosterone concentrations were achieved quickly whereas values within the male reference range were reached seven to nine days after injection. In FtMs receiving a 125 mg depot, testosterone concentrations peaked at day 0.5 and decreased towards the lower limit of

the reference range at the end of the two weeks, 1 out of 4 patients reached a level below the reference range. Testosterone levels peaked at day 0.5 or day 1 in patients receiving 250 mg per 4 weeks with a decrease below reference values after two weeks in all four patients. The calculated areas under the curve (AUC) were: 7349 (7111 – 8262) and 10596 (8974 – 15923) (median (range)) for the 125 mg/2 weeks and 250 mg/4 weeks group, respectively. For an equal time period of 4 weeks, the average AUC of the group treated biweekly would, by consequence, amount to 14698 or 1.39 times the AUC of the group treated with a single injection.



**FIGURE 2:** Salivary testosterone profiles of four individuals receiving 125 mg TEM/2 weeks (left panel A) and four individuals receiving 250 mg TEM/4 weeks (right panel B). Data points of day 0 were sampled directly before injection. Dashed line represents the upper and lower reference values for healthy male subjects (135-400 pmol/L). Note the logarithmic scale on the y-axis.

## DISCUSSION

The first objective of this study was to develop a method to measure salivary testosterone concentrations. An ID-LC-MS/MS method was therefore developed and thorough analytical validation showed high accuracy and sensitivity. The LOQ of the described method (approximately 1 fmol (0.3 pg)) compares favorably, in terms of sensitivity, to other published methods [5-7], except Turpeinen, *et al.* measures salivary testosterone more sensitive [8].

The accuracy of serum testosterone measurements by immunoassays at low concentrations has been subject to discussion [9]. As testosterone levels in saliva are approximately 50 times lower than in serum, the accuracy of immunoassays for salivary testosterone is likely to be even more compromised [10]. Salivary testosterone measurements has therefore not been widely implemented into routine diagnostics and only limited into research, even though sample collection is minimally invasive and can be carried out in diverse populations and settings. To warrant the accuracy of



the described ID-LC-MS/MS method, and in the absence of a reference method or standard for salivary testosterone measurement, the standard solution was calibrated by a serum total testosterone ID-LC-MS/MS method that was concordant with a published reference method, gas chromatography – mass spectrometry [4]. The introduction of the ID-LC-MS/MS technique for accurate and sensitive testosterone measurements enables us to do new types of quantitative and qualitative research.

Furthermore, this ID-LC-MS/MS method was used to determine the adult male salivary testosterone reference interval. Even though the healthy volunteers were not physically and biochemically examined, the result, i.e. 135 to 400 pmol/L, is similar to male reference values reported by others [5;6;11].

The second aim of this study was to use this method to evaluate the profile of testosterone in saliva in FtM transgender adolescents after TEM injection. A similar testosterone profile was shown in all FtM adolescents: testosterone levels peaked supra-physiologically directly after injection, and decreased to reach reference levels seven to nine days later. All subjects receiving 250 mg TEM per 4 weeks showed nadir concentrations below the reference values in week 3 and 4. The reason for measuring testosterone concentrations in the inter-injection period was to investigate if nadir concentrations might be the cause of complaints about less energy and mood swings by some FtM adolescents. Indeed testosterone concentrations below the reference range were seen in the subjects receiving 250 mg depots per 4 weeks. However, also the major difference between the peak and the relatively low nadir two weeks after the TEM injection might play a role in the discomfort of the FtMs. The total exposure to testosterone as judged by the areas under the curve would be 39% higher in the group treated with two injections of 125 mg per month as compared to the patients receiving a single monthly injection. It would be interesting to evaluate this in a larger group and to include a psychological assessment to see whether patients using TEM per 4 weeks without any complaints at the end of the 4 weeks period have higher salivary testosterone levels compared to patients with complaints.

The use of intra-muscular testosterone injections has been evaluated in different patient-groups [12;13]. These studies also show a supra-physiological testosterone peak after injection, which is in agreement with our results. However, serum total testosterone was measured in these studies in contrast to our study where testosterone was measured in saliva. The median salivary testosterone peak value found in our study is approximately 5-fold higher than the upper limit of the male reference range, whereas the peaks found in studies based on total testosterone in serum are less elevated [12;13]. Administration of testosterone will result in increased total serum testosterone levels, but not necessarily in higher concentrations of binding proteins. By contrast, we expected to see and indeed observed a decrease in serum sex hormone-binding globulin (SHBG) levels when patients start cross-hormone therapy (data not shown). The free fraction of testosterone in serum might therefore be

relatively more elevated. As salivary testosterone concentrations mirror the serum free testosterone concentrations, salivary testosterone may be more reflective of the physiological state in the study population. To gain more insight into the difference in the elevated testosterone levels in serum versus saliva, an investigation of the physiological state of this study population with paired serum and saliva samples included could shed more light on this issue.

Due to obvious ethical reasons, pharmacological studies are often performed in adults. However, the results and conclusions of these studies are also applied in vulnerable groups such as children and adolescents without further research, which is also the case with the use of TEM. This study was performed in adolescents and even though the results comply with the findings in adults by other researchers, it is a priority to offer young patients less invasive therapy by i.e. lowering the frequency of invasive TEM injections while maintaining testosterone levels within the normal range. By measuring the testosterone profile in saliva, we can establish the balance between the invasiveness of TEM therapy and reaching the goals of normal testosterone levels. Both the pediatricians and the patients experienced the procedure as a practical and non-invasive approach for sequential measurements. Since patients can collect saliva at home without the assistance of medical personnel, the timing of sampling is flexible and not restricted to working hours.

The relationship between testosterone levels both above and below the reference range and patient health and wellbeing is a topic that warrants further research. Although many studies to date suggest that short-term supra-physiological levels of testosterone, as occurring post-injection during treatment of FtM adolescents, have minimal adverse effects (no increase in mortality, breast cancer, vascular disease, or other major health problems were reported) [14], high levels of testosterone are associated with violent behavior in man [15] and with adverse behavioral and psychiatric effects (including euphoria, depression, anxiety, and paranoia) in animals [16]. Therefore, the main objective of testosterone administration should be to reach and maintain testosterone levels within the male reference range. The salivary testosterone profile of TEM injections shows high inter-individual variability, additional study is needed to established whether this correlates with the patients' discomfort. If so, this calls for personalized dosing if the patient shows signs of discomfort and testosterone levels are evaluated. Alternative therapies may also be considered: for instance, testosterone undecanoate and testosterone gels show a more gradual and favorable pharmacokinetic profile [13;17]. Testosterone gels are already used by some FtM adults to maintain secondary sexual characteristics, however there is no experience with this therapy in FtM adolescents. For adults, we support the suggestion by Gooren [17] that the choice of treatment should ultimately come down to the wishes of the patient.

## CONCLUSION

In conclusion, we developed a sensitive and accurate ID-LC-MS/MS method for measuring salivary testosterone, which allowed us to monitor the testosterone profile after TEM injections in FtM transgender adolescents in a patient-friendly and non-invasive manner. TEM injections produce a quick increase in testosterone to highly supra-physiological levels which subsequently decrease and, after approximately three weeks, reach sub-physiological levels. Further investigation including paired serum and saliva samples, and psychological assessment in a larger study population is needed to establish whether salivary testosterone can be used to monitor pharmacokinetics of testosterone therapy.

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