A Time-Resolved Fluorescence Immunoassay for the Measurement of Testosterone in Saliva: Monitoring of Testosterone Replacement Therapy with Testosterone Buciclate

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Monitoring of testosterone replacement therapy requires a reliable method for testosterone measurement. Determination of salivary testosterone, which reflects the hormone's biologically active plasma fraction, is a superior technique for this purpose. The aim of the present study was to establish a new sensitive time-resolved fluorescence immunoassay for the accurate measurement of testosterone levels in saliva and to validate it by monitoring testosterone replacement therapy in eight hypogonadal men.

A clinical phase I study with the new ester testosterone buciclate was performed to search for new testosterone preparations to produce constant serum levels in the therapy of male hypogonadism. After two control examinations eight male patients with primary hypogonadism were randomly assigned to two treatment groups (n=2x4) and given single doses of either 200 mg (group I) or 600 mg (group II) testosterone buciclate intramuscularly. Saliva and blood samples were obtained 1, 2, 3, 5 and 7 days post injection and then weekly for three months.

The time-resolved fluorescence immunoassay for salivary testosterone shows a detection limit of 16 pmol/l, an intra-assay CV of 8.9% (at a testosterone concentration of 302 pmol/l), an inter-assay CV of 8.7% (at a testosterone concentration of 305 pmol/l) and a good correlation with an established radioimmunoassay of r=0.89. The sample volume required by this method is only 180 µl for extraction and duplicate determination. The assay procedure requires no more than three hours.

In group I (200 mg) testosterone did not increase to normal levels either in saliva or in serum. However, in group II, androgen levels increased significantly and were maintained in the normal range for up to 12 weeks with maximal salivary testosterone levels of 303 ±18 pmol/l (mean±SE) and maximal testosterone levels of 13.1 ±0.9 pmol/l (mean±SE) in serum in study week 6 and 7.

The time-resolved fluorescence immunoassay for salivary testosterone provides a useful tool for monitoring androgen status in men and women and is well suited for the follow-up of testosterone replacement therapy on an outpatient basis. The long-acting ester testosterone buciclate is a promising agent for substitution therapy of male hypogonadism and in combination with testosterone monitoring in saliva offers an interesting new perspective for male contraception.

Key words: Testosterone; Saliva; Testosterone buciclate; Fluorescence immunoassays; Biotin.

Introduction

In monitoring the function of steroid-producing organs, the quantification of steroid hormones in saliva is a useful, non-invasive approach for the assessment of biologically active hormone concentrations (1-3). The appearance in saliva of steroids such as testosterone is flow rate-independent and is caused by their free diffusion from blood plasma to saliva through the epithelial cells of the salivary gland (2, 4, 5), which is comparable with the physiological process of entering target cells. It is likely, therefore, that the non-sex hormone-binding globulin (SHBG)-bound fraction is readily available to target cells physiologically active, and that this fraction is identical with salivary testosterone (6-8). In plasma, the non-SHBG-bound fraction mainly represents the unbound fraction (1-3 %) and the albumin-bound hormone fraction (20-80 %) in plasma (9, 10). Because of the non-invasive and stress-free technique of sample collection as well as the ease of multiple sampling by the patient himself, salivary hormone assays are being used increasingly in psychological (11, 12), anthropological (13, 14), and field studies (15). From a clinical point of view, salivary testosterone results not only seem to be of greater diagnostic use in hirsutism than any of the currently used serum androgen parameters (16), but also seem to be well suited for monitoring testosterone replacement therapy.

Traditionally, salivary testosterone has been measured by immunoassays using 3H- or 125I-labelled testosterone-derivatives as tracers. In order to exploit the exceptional affinity of the avidin-biotin system (K = 1015 M-1) (17), a low molecular weight testosterone-biotin conjugate for use as a tracer in a testosterone immunoassay was synthesized. With this stable tracer as a tool, a time-resolved fluorescence immunoassay (TRFIA) was established as a new, non-isotopic approach to the measurement of salivary testosterone.

Commonly used testosterone esters, given either intramuscularly or orally, correct many symptoms of testosterone deficiency, but their pharmacokinetics are...
still unfavourable. The potential application of androgens to substitution therapy of aging men (18) and to the surveillance of male contraception (19, 20), however, makes the increasing use of androgen preparations likely. To assess the pharmacokinetics and pharmacodynamics of the new WHO and National Institute of Health (NIH) ester, testosterone buciclate, in hypogonadal men, a clinical phase I-study was performed (21, 22).

This study was monitored by the measurement of salivary testosterone with the newly established TR-FIA.

**Subjects and Methods**

Establishment and validation of the TR-FIA for the measurement of salivary testosterone

Synthesis of a testosterone 3-(O-carboxymethyl)-oxime (testosterone-3-CMO)-biotin conjugate

Fifty µmol (18.075 mg) of testosterone-3-CMO (Sigma, Deisenhofen, Germany, Cat. No. T-8390) were dissolved in 200 µl of dry, amine free N,N′-dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany, Cat. No. 2952). Equimolar amounts (6.31 mg and 11.18 mg respectively) of N-hydroxysuccinimide (NHS) (Sigma, Cat. No. H-7377) and N,N′-dicyclohexylcarbodiimide (Sigma, Cat. No. D-3128) were dissolved each in 50 µl of DMSO and added to the testosterone-3-CMO. This reaction mixture was left at room temperature in the dark for 24 hours during which time the testosterone-3-CMO was converted into its NHS ester, and needle-like crystals of dicyclohexyl urea appeared. For coupling of the testosterone-N-hydroxysuccinimide (NHS) (Sigma, Cat. No. H-7377) and N,N′-dicyclohexylcarbodiimide (Sigma, Cat. No. D-3128) were dissolved each in 50 µl of DMSO and added to the testosterone-3-CMO. This reaction mixture was left at room temperature for 24 hours in the dark. The proposed mechanism of reaction and structure of the final product are shown in Figure 1 (24). The course of the reaction was monitored by thin layer chromatography under UV-light.

Purification of the testosterone-3-CMO-biotin conjugate

Aliquots of this reaction mixture were applied to a 10 x 400 mm Trisacryl GF 05 M (Serva, Heidelberg, Germany, No. 67010) column after 1:10 (v/v) dilution in column buffer (50 mmol/l Tris-HCl adjusted to pH 7.8 with 1 mol/l HCl) using fast performance liquid chromatography (FPLC). The elution pattern of the column showed two well-separated peaks.

Characterization of the testosterone-3-CMO-biotin conjugate

The testosterone content of the fractions was analyzed by a commercially available radioimmunoassay (RIA-mat Testosteron, Byk Sangtec Diagnostica GmbH, Dietzenbach, Germany, No. 322.451). Testosterone concentrations corresponded to the absorbance at 254 nm of the column eluate. In addition the fractions were tested as tracer for maximal binding and displacement by a 6.93 nmol/l testosterone standard. Determinations were performed in duplicate, and the ratio between the signal obtained at maximal binding and the displacement by the 6.93 nmol/l standard was calculated. The second of the two peaks at 254 nm showed the highest binding ratio and apparently contained the testosterone-biotin conjugate, while the first peak contained mainly uncoupled testosterone. Finally, the biotin content of the fractions was measured with an in-house TR-FIA for biotin determination and confirmed the previous findings by showing significant amounts of biotin in the second peak of the elution pattern.

Testosterone antiserum

Testosterone antiserum was kindly provided by Prof. F. Bidlingmaier (Bonn, Germany). Specificity of the antiserum was determined by addition of increasing amounts of cross-reacting steroids as the unknown sample in the immunoassay described here. The doses of testosterone and cross-reacting steroid hormone effectively displacing 50% of the tracer from the antibody (ED-50) were compared and the respective concentrations were expressed as ED-50 (testosterone): ED-50 (cross-reacting steroid) x 100 (%).

Fig. 1 Pathway of synthesis and proposed chemical structure of the testosterone-biotin tracer conjugate.
Saliva sampling

Saliva samples were taken at two control examinations, shortly before and 1 hour and 1, 2, 3, 5, 7 days after testosterone buciclate injection, and then weekly up to week 16. Of three existing types of saliva sampling devices (“Salivette”, Sarstedt, Nümbrecht, Germany) only one type (No. 51.1534.002) can be used without a positive bias in the analytical results (25, 26). This type of “salivette” consists of a 40 x 9 mm non-glued polyester wool swab wrapped in polypropylene film, an inner and an outer polystyrene tube. For sampling, ten minutes after rinsing the mouth with water (but no tooth brushing, to avoid gingival microlesions) (27), the polyester wool swab is kept in the mouth or chewed on for 0.5–2 min. The swab is then transferred to the inner of the two polystyrene tubes and the sample is frozen. Unpreserved saliva samples stored unrefrigerated have been shown to yield satisfactory results for male subjects, but lead to increased measured concentrations for female subjects (28). In our own experiments a storage time up to three months at room temperature did not have significant influence on the salivary testosterone results. Storage time of frozen samples does not seem to affect the reliability of the measurement of salivary steroids (25). By centrifugation of the thawed samples prior to analysis, the salivary liquid is transferred to the outer tube. Saliva samples which on inspection are contaminated with blood are excluded from the analysis (29).

Assay design

Affinity purified swine anti-rabbit immunoglobulin (Dako, Hamburg, Germany, No. Z 400) was immobilized on to microtitre plates (Maxisorp, Nunc, Roskilde, Denmark, No. 4-42404) at 200 ng per well in 200 µl of 50 mmol/l trisodium phosphate adjusted to pH 9.6 with 1 mol/l HCl. These plates can be stored at 200 ng per well in 200 µl of 50 mmol/l trisodium phosphate buffer (DELFIA platewash, Pharmacia, No. 1296–001).

Reference radioimmunoassay

The established RIA for salivary testosterone, with which the TR-FIA was compared, requires 1 ml to 3 ml saliva for extraction with ether and salivary testosterone determination (31).

For the RIA an anti-testosterone-3-CMO-BSA-serum was used and the only steroid which significantly cross-reacted was 5α-dihydrotestosterone (60%). Sensitivity of the saliva RIA, taken as the value on the standard curve at two standard deviations below the maximum specific binding, was 35 pmol/l. The intra- and inter-assay coefficients of variation were 3.7% and 13.8%, respectively.

Sample preparation

In order to eliminate matrix interference, saliva samples (180 µl) were extracted by addition of 1.8 ml of dichloromethane (Merck, Darmstadt, Germany) containing 50 µg/ml polyethylene glycol 10000 (Merck, Darmstadt, Germany). Samples were mixed for 15 minutes on a horizontal shaker. The samples were then left unagitated for 10 minutes to allow phase separation. Subsequently, 1.65 ml of the organic solvent was carefully removed and evaporated using a water bath and the residue was redissolved in the “artificial saliva” (165 µl). Recovery of this extraction step was determined by measurement of control samples containing a known amount of tritium-labelled testosterone (Bio Merieux, Nümbrecht, Germany) in a µ-scintillation counter (Berthold, Bad Wildbad, Germany).

Blood samples

Blood samples for hormone determinations were drawn between 8.00 and 10.00 h at two control examinations, shortly before and 1 h and 1, 2, 3, 5, 7 days after testosterone buciclate injection, and then weekly up to week 16. Blood samples for hormone determinations were separated at 800 x g and stored at -20 °C until assayed.

Imunoassay serum

SHBG was determined by highly specific fluoroimmunoassay (DELFIA SHBG, Pharmacia GmbH, Freiburg, Germany) (22, 32). The detection limit for SHBG was 6.3 nmol/l. The intra- and inter-assay coefficients of variation for SHBG were 5.3% and 7.2%. In our laboratory the normal range for adult males for SHBG is 11–71 nmol/l. Testosterone and dihydrotestosterone were separated in extracted serum samples by high performance liquid chromatography before measurement by RIA, as described earlier (33). The detection limits for testosterone and dihydrotestosterone were 0.28 and 0.14 nmol/l, respectively. The intra- and inter-assay coefficients of variation for testosterone were 6.8% and 9.8%, respectively, and for dihydrotestosterone 8.5% and 13.4%, respectively. The lower normal limit for androgens (sum of testosterone and dihydrotestosterone) after separation by high performance liquid chromatography was 10 nmol/l and the upper normal limit for dihydrotestosterone is 2.9 nmol/l in our laboratory.
Patients and medication

The study protocol of the phase I study with testosterone buciclate was approved by the Steering Committee of the WHO Task Force on Methods for the Regulation of Male Fertility, by the WHO Toxicology Group, by the WHO Scientific and Ethical Review Group and by the WHO Secretariat Committee on Research Involving Human Subjects, as well as by the Ethics Committee of the University of Münster and the State Medical Board. Eight adult patients (28–48 years) with primary hypogonadism without additional diseases gave informed consent and were enrolled in the study. After two control examinations the patients were randomly allocated to either of two treatment groups. Two-hundred milligrams of testosterone buciclate (0.8 ml injection vol, one injection site) were administered by injection to the medial glutel muscle on day 0 to study group I (n=4; age, 37.8 ± 8.2 years; body weight, 78.2 ± 12.6 kg; height, 181.7 ± 5.8 cm). Similarly, 600 mg testosterone buciclate (2.4 ml injection vol, one injection site) were given to study group II (n=4; age, 32.3 ± 3.4 years; body weight, 84.7 ± 7.8 kg; height, 183.6 ± 6.0 cm).

The androgen ester testosterone buciclate was prepared by Palmer Research (Holywell, Great Britain) under WHO auspices under Good Manufacturing Practice conditions. After wet milling of crystalline testosterone buciclate obtain at least 75% of particles in the size range of 10–50 µm, the drug was sterilized by γ-radiation and suspended in sterile aqueous solution. The suspension was shaken vigorously for 1 minute before injection. The batch used for all injections had a concentration of 250 g/l testosterone buciclate, which corresponds to a concentration of 158.6 g/l (0.55 mol/l) unesterified testosterone. The clinical data and hormone serum levels of this study have been published elsewhere (21).

Statistical methods

Variation over time and differences between study groups were evaluated by multifactorial analysis of variance for repeated measures. Computations were performed using statistical software Statview® for Macintosh, version D-4.5, statistical software package SPSS/PC+, version 4.0 (SPSS, Inc., Chicago, IL, USA) and Statgraphics Plus, version 5.2 (STSC, Inc., Rockville, MD, USA). When necessary, variables were logarithmically (log) transformed before analysis to achieve normal distribution and triplicate determination, and the assay is easy to handle and takes 6 hours for the determination of 80 samples, including the sample extraction. The testosterone-biotin tracer is stable for at least three years.

Results

Assay characteristics of the new TR-FIA for salivary testosterone

The new TR-FIA requires as little as 180 µl of saliva for extraction and triplicate determination, and the assay is easy to handle and takes 6 hours for the determination of 80 samples, including the sample extraction. The testosterone-biotin tracer is stable for at least three years.

Specificity

The displacing potency of potentially cross-reacting steroids was investigated by addition of increasing doses of steroids as the unknown sample in the immunooassay procedure, and comparison of ED-50. In this assay, α,α-dihydrotestosterone had a relative displacing potency of 19.5%, androstenedione 9.7%, 6α-hydroxytestosterone 6.2%, and 11α-hydroxytestosterone 3.6%.

Sensitivity

The sensitivity of the TR-FIA was defined as the intercept with the displacement curve of the mean minus two standard deviations of a determination of the zero standard (n=30). The sensitivity was 16 pmol/l.

The mean 50% intercept of a typical displacement curve was 220 pmol/l.

Imprecision

The intra-assay coefficients of variation were determined by a measurement of three different saliva samples (n=36) with testosterone concentrations of 302.0 pmol/l, 158.5 pmol/l and 125.0 pmol/l. The intra-assay imprecision for the salivary testosterone concentrations of 302 pmol/l, 158.5 pmol/l and 125.0 pmol/l (n=36) was 8.9%, 14.6% and 11.4%, respectively.

Two different samples were determined in 24 consecutive assays for calculation of the inter-assay imprecision. The inter-assay coefficient of variation for the concentrations of 182.4 pmol/l and 304.8 pmol/l was 7.9% and 8.7%, respectively (n=24).

Recovery

The mean recovery of the extraction step was 93% (for n=12).

Salivary samples with testosterone concentrations of 236.5 pmol/l, 210.5 pmol/l, 182.0 pmol/l and 131.1 pmol/l were serially diluted with zero-standard. The measured concentrations were compared with the expected concentrations and expressed as a percentage of the expected value. The mean recoveries were 104.1%, 94.2%, 93.6%, and 95.6%, respectively.

Correlation of salivary testosterone TR-FIA with RIA

Salivary samples (n=132, concentration range: 37 pmol/l – 403 pmol/l) from healthy men and women were analyzed using both the established RIA adapted for salivary testosterone measurement and the new TR-FIA. The correlation found was significant (r= 0.89, p<0.0001) (Fig. 2)
Monitoring of testosterone replacement therapy in hypogonadal men in saliva

After injection of 600 mg testosterone buciclate, salivary testosterone increased from $203 \pm 36$ pmol/l (mean ± SE) to $303 \pm 18$ pmol/l (mean ± SE) and was maintained in the normal range ($230$ pmol/l – $570$ pmol/l as validated for the TR-FIA) for up to eleven weeks. However, after injection of 200 mg testosterone buciclate, salivary testosterone did not increase significantly. The time course of increase of salivary testosterone was parallel to that of serum androgen levels for both groups. (Fig. 3)

Correlation of salivary testosterone concentration with serum SHBG, oestradiol, dihydrotestosterone and testosterone

The salivary testosterone levels measured after injection of testosterone buciclate showed a negative correlation with SHBG in serum ($r=0.50$; $p<0.001$), a positive correlation with serum oestradiol ($r=0.52$; $p<0.001$), a positive correlation with serum dihydrotestosterone ($r=0.37$; $p<0.001$) and a weak, but significant, correlation with total serum testosterone ($r=0.22$; $p=0.009$).

Discussion

We have established a new method for the measurement of salivary testosterone and used it for the monitoring of testosterone replacement therapy with the androgen ester testosterone buciclate in eight hypogonadal men.

When multiple sampling is required, as in the monitoring of testosterone replacement therapy, salivary hormone measurement is non-invasive and stress-free. The methods are accurate and reliable. (34). Frequent collecting and mailing of saliva samples is practicable for the patients and does not require assistance of medically trained staff (25).

The newly established TR-FIA is characterized by a number of advantageous features. A sample volume of not more than $180 \mu$l of saliva was sufficient for the accurate measurement of salivary testosterone, while comparable methods (28, 31) require a sample volume between $500 \mu$l and $5000 \mu$l. The non-isotopic method is easy to perform, inexpensive, takes a total of three hours incubation time and has a lower detection limit of $16$ pmol/l. Sensitivity and accuracy of this assay should allow the measurement of salivary testosterone in women and even pre-pubertal children. Correlation with an established assay was good ($r=0.89$). A slope of the regression line of only $b=0.51$ is most probable due to the use of different standards. The employed biotintestosterone conjugate has a molecular size weight of 740.5 Da which is comparable with the analyzed steroid (288.4 Da) and can be stored for more than 24 months without significant loss of activity. The use of biotin as a primary probe provides a signal amplification system (23) and also has the advantage of a versatile endpoint determination: this tracer can be detected using EIA readers, TR-flurometers, luminometers or in conventional gamma-counting, thereby reducing between-laboratory variance. The antibody used is highly specific. The assay procedure is simple, reliable and rapid.

Testosterone in serum is largely bound to SHBG and albumin. The unbound testosterone and corticosteroid-binding globulin-bound testosterone fractions are minimal. The unbound testosterone is biologically active, while SHBG-bound testosterone is not. The bioavailability of albumin-bound testosterone is controversial (35–37), but some studies suggest that bioavailable testosterone includes circulating free testosterone and a large proportion of albumin-bound testosterone (6, 38). An argument in favour of this theory is that the half-dissociation time of testosterone from SHBG is relatively long (>20 sec), while in contrast, the half-dissociation time of testosterone from albumin (<1 sec) is within the probable capillary transit time. Therefore these fractions may be available to target tissues and saliva, respectively. Salivary testosterone levels are independent of salivary flow rate (5) and, due to the rapid passage of steroid hormones from blood to saliva, despite enzymes such as 5α-reductase being present in salivary glands (39), steroid metabolism is probably not an important source of error in the measurements of testosterone in saliva (40). The importance of metabolism of testosterone in salivary glands is still unclear (41, 42), but recent results of in vitro experiments (40) and the excellent correlation of salivary tes-
testosterone with total-, free- and particularly non-SHBG-bound testosterone (16), suggest that metabolism of steroids in human salivary glands does not affect the level of salivary testosterone. Thus, salivary testosterone appears to reflect the biological activity of the hormone.

Development of accurate assays for analytes present at low picomolar concentrations in body fluids is a demanding task. The appearance of an unexpected positive bias in salivary testosterone assays which was dependent on the material chosen for salivary sampling is an example of the encountered difficulties. In this context we could confirm earlier results of Dabbs (28), who detected testosterone-like immunoreactivity in cotton wool swabs used in saliva sampling devices. In search for an optimal method of salivary sampling for the testosterone TR-FIA, the eluates from three of the existing types of salivettes were measured. These were soaked with 1 ml of saline under sterile conditions, frozen, thawed and centrifuged as described above for the pretreatment of salivary testosterone samples. In two of the extracted eluates we found testosterone concentrations in a range corresponding to normal male levels in saliva. The testosterone-like immunoreactivity was detectable even after organic extraction, supporting the hypothesis that the nature of this immunoreactivity is similar to that of other steroid hormones. Only one type of salivette, which did not contain glue and was not prepared with citric acid for the stimulation of salivary flow, did not demonstrate significant testosterone-like immunoreactivity.

In further experiments we compared this sampling method to direct sampling in a glass tube. Forty-eight medical students tested both sampling methods, one method immediately following the other. The sampling method using the salivettes led to lower matrix effects (i.e. contamination with food particles) than the direct collecting of saliva in a glass tube. In addition, most of the volunteers felt the salivettes to be a more comfortable and more hygienic sampling method.

Up to now the achievement of constant serum levels in a physiological range has been the main pharmacokinetic goal when searching for new testosterone preparations. Salivary testosterone reflects the bioactive testosterone fraction (1, 2, 38, 43, 44). The measurement of this variable is of interest as a meaningful criterion for the effectiveness of an androgen preparation.

Due to the close link between sperm production and male hormones, endocrinological suppression of sperm production is parallel with suppression of androgen production. Thus androgen replacement therapy plays an important role in the development of methods for male contraception.

This study confirms both the adequacy of measurement of salivary testosterone as a monitoring system and testosterone buciclate as an androgen ester with desirable pharmacokinetics for testosterone replacement therapy: after administration of testosterone buciclate the course of the salivary testosterone curve and the serum testosterone curve behaved similarly. Both salivary testosterone and serum testosterone increased to normal levels and were maintained in a physiological range for seven weeks after a single injection of 600 mg of testosterone buciclate. After administration of 200 mg of testosterone buciclate, however, salivary testosterone levels as well as serum testosterone levels did not increase (Fig. 2).

A negative correlation of salivary testosterone with serum SHBG reflects the important role of circulating SHBG on the amount of biologically active testosterone and salivary testosterone. According to the thesis that only the non-SHBG-bound testosterone is bioactive and reflected by salivary testosterone, high SHBG levels are associated with low salivary testosterone levels and vice versa. Furthermore, a positive correlation between salivary testosterone and serum oestradiol, which is one of the co-regulators of SHBG synthesis in the liver, could be explained by a displacement of bound androgens by increasing levels of oestradiol and vice versa.

In summary, we have established a new method for the measurement of testosterone in saliva using the streptavidin-biotin tracer as a tool. The new ultrasensitive method is fast, non-isotopic and requires a sample volume of not more than 180 µl for extraction and duplicate determination. In addition, the new TR-FIA was applied to a clinical phase I study, in which the new long-acting androgen ester, testosterone buciclate, was tested as a substitution therapy for male hypogonadism. Its favourable pharmacodynamics and pharmacokinetics, as shown previously by monitoring testosterone in serum (21), were confirmed by monitoring its bioactive fraction by multiple consecutive measurements of salivary testosterone.

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References


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