# An improved method for measurement of testosterone in human plasma and saliva by ultra-performance liquid chromatography-tandem mass spectrometry

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## Abstract

The aim of the study was to develop and validate a practical assay of clinically relevant testosterone levels in human plasma and saliva. We performed ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis on Atlantis dC18 steel column using a mobile phase of 2-mM ammonium acetate and acetonitrile (20:80, v: v) that was delivered at 0.3 ml/min. After adding d3-testosterone as an internal standard (IS), we extracted plasma and salivary samples with methyl tert-butyl ether. Mass spectrometry was performed in electrospray positive-ion mode. Targeted ion transitions were examined at m/z 289.18 → 97.04 and 292.24 → 97.04 for testosterone and IS, respectively. We validated the method according to the US Food and Drug Administration guidelines. Elution times for testosterone and IS were both around 1.35 min. Testosterone level was linearly associated ($r^2$ = 0.9975 and 0.9958) with peak area ratio of testosterone to IS between 0.5–50 ng/ml and 10–400 pg/ml in plasma and saliva, respectively. The coefficient of variation and bias were ≤12.6% and ≤±12.1% in plasma and ≤10.2% and ≤±5.3% in saliva. The extraction recovery of testosterone was ≥92% from plasma and ≥94% from saliva. Testosterone was stable (≥91%) for 24 h at room temperature and for 8 weeks at −20°C in both plasma and salivary samples. We report a simple, validated, UPLC-MS/MS assay that can be used to determine clinically relevant levels of testosterone in human plasma and saliva.

**Key words:** Artificial saliva, d3-testosterone, human plasma, testosterone, ultra-performance liquid chromatography-tandem mass spectrometry

## INTRODUCTION

Testosterone (CAS: 58-22-0) is required to develop and maintain reproductive tissues and sexual characteristics in men.[1,2] Its excess in women causes hirsutism and virilism and may indicate the presence of gonadal or adrenal tumors.[3,4] Further, testosterone is widely used in the treatment of hypogonadism.[5] Thus, determining testosterone level is important in the management of sex hormonal disorders in both men and women.[6]

Nearly 98% of testosterone in plasma is "inactive" because it is protein bound mainly to sex hormone-binding globulin and albumin, and about 1%–2% is unbound and bioavailable

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or "active." Nevertheless, total plasma testosterone level is commonly used clinically due to lack of reliable available free testosterone commercial assays.[7]

Measurement of testosterone level in human saliva has attracted increasing interest because it reflects the unbound, bioavailable plasma testosterone; it permits obtaining samples at short intervals even from pediatric patients; and it does not require venipuncture, medical personnel, or laboratory facilities for collecting and storing samples.[8,9]

Several factors influence testosterone level, including sex, age, health, and sample collection time.[7] Thus, different reference ranges have been reported in various groups. For healthy men, the reference range is 3.8–9.5 ng/ml in plasma and 30–130 pg/ml in saliva.[10,11]

Several methods for measuring testosterone level have been reported. These include enzyme immunoassay,[10,12] radioimmunoassay,[13,14] high-performance liquid chromatography,[15,16] and liquid chromatography-tandem mass spectrometry.[11,17-19] Radioimmunoassay, the most commonly used method, has high sensitivity but suffers from cross-reactivity and relatively low specificity.[20]

We report a specific, sensitive, and fast ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay for determining clinically relevant testosterone level in human plasma and saliva. The stability of testosterone in extracted and unextracted plasma and salivary samples was tested under common clinical laboratory conditions.

## EXPERIMENTAL

### Chemicals and reagents
Testosterone, d3-testosterone, as well as ammonium acetate, acetonitrile, and methyl tert-butyl ether were purchased from Sigma-Aldrich MO, USA; IsoScience, PA, USA; and Fisher Scientific, NJ, USA, respectively. The Research Ethics Committee of King Faisal Specialist Hospital and Research Center (KFSHRC) approved the study. We procured human plasma from KFSHRC blood bank.

### Equipment and analytical conditions
The UPLC-MS/MS consisted of Xevo-TQD detector with an atmospheric pressure ionization interface, Acquity UPLC H-Class system, and integrated solvent and sample manager (Waters Corporation, Milford, MA, USA). We performed the analysis at room temperature by means of reversed-phase Atlantis dC18 (2.1 mm × 100 mm, 3 μm) steel column, protected by an online guard filter (0.2 μm × 2 mm). The mobile phase was made of 2-mM ammonium acetate (pH = 4.2 ± 0.1, adjusted with formic acid) and acetonitrile (20:80, v: V) and was delivered at 0.3 ml/min. The run time was 2.5 min. We operated the electrospray

ionization source at a capillary voltage of 1.5 kV (positive-ion mode) and a cone voltage of 75 V. We used nitrogen and argon as nebulizing/desolvation gas (1000 l/h) and collision gas, respectively, and maintained pressure of the cell at 3.6 E$^{-003}$ mbar. We applied an optimum collision energy of 26 eV and dwell value of 0.02 s for testosterone and the internal standard (IS). We maintained the ion source at 150°C and desolvation temperatures at 500°C. We quantified testosterone and the IS in the positive-ion mode at mass to charge (m/z) 289.18 → 97.04 and 292.24 → 97.04 for testosterone and the IS, respectively. We used Mass lynx Ver 4.1 (Waters Corporation, Milford, MA, USA) program to control instrument parameters, acquire data, integrate and smooth peaks, and measure signal-to-noise ratios.

### Standard and control samples
We made stock solutions of testosterone and IS in methanol (1.0 μg/ml). We used human plasma and artificial salivary samples to prepare quality control (QC) and standard samples. Artificial saliva was prepared as previously described.[21] Eight calibration standards ranging from 0.5 to 50 ng/ml for plasma and from 10 to 400 pg/ml for saliva and four QC samples (0.5, 1.5, 25, and 45 ng/ml for plasma and 10, 30, 180, and 380 pg/ml for saliva) were used for validation purposes. After vortexing for 1 min, we transferred 0.5-ml plasma and 1.0-ml salivary samples to 7-ml glass culture tubes that were stored at −20°C. We prepared IS working solutions (50 ng/ml for plasma and 200 pg/ml for saliva) in methanol.

### Plasma and salivary sample
We added the IS (100 μl of 50 ng/ml or 200 μl of 200 pg/ml in methanol for the plasma and salivary assays, respectively) to 7-ml glass culture tubes containing either 0.5 plasma or 1.0-ml saliva and vortexed the tubes for 30 s. We then added 5 ml of methyl tert-butyl ether, vortexed the mixture for 4 min, and centrifuged it at 4600 rpm for 15 min (20°C). The supernatants were transferred to borosilicate tubes to dry under a nitrogen stream (room temperature). We reconstituted the residue in 100-μl mobile phase and injected 10 μl of the clear solution into the system.

### Extraction recovery
We determined extraction recovery by comparing peak area of four samples (0.5, 1.5, 25, and 45 ng/ml for plasma and 10, 30, 180, and 360 pg/ml for saliva) that were prepared according to the method and compared with the peak area of the corresponding samples prepared in mobile phase and then spiked on previously extracted blank plasma or saliva. We determined IS extraction recovery similarly but at a level of 50 ng/ml for plasma and 200 pg/ml for saliva.

### Effect of matrix
We evaluated the matrix effect by comparing peak area response obtained from testosterone at four concentrations (0.5, 1.5, 25, and 45 ng/ml for plasma and 10,

30, 180, and 360 pg/ml for saliva) prepared in mobile phase to the peak area response obtained from the corresponding samples spiked on previously extracted blank plasma or saliva.

## Calculations

To account for endogenous testosterone levels in blank plasma, we subtracted the peak area ratio that was observed with the blank plasma from the peak area ratios that were observed with the spiked samples and regressed the difference against nominal testosterone level.

## Stability studies

Two QC samples (1.5 and 45 ng/ml for plasma and 30 and 360 pg/ml for saliva) were used for stability studies. For each sample, five aliquots were processed and immediately analyzed (baseline), five were stored at room temperature (24 h) before processing, and five were placed at −20°C (8 weeks) prior to processing. Further, five aliquots were placed at room temperature (24 h) or −20°C (48 h) after processing and before analysis. Finally, 15 aliquots were placed at −20°C for 24 h and then at room temperature until completely thawed. Of those, five were analyzed, and the others were placed at −20°C, repeating the cycle two more times.

## RESULTS AND DISCUSSION

## Method development

To improve UPLC conditions, 2-mM ammonium acetate (pH = 4.2)/acetonitrile (20:80, v: V) was used as a mobile phase at room temperature under isocratic condition. This reduced the run time to 2.5 min (compared to 3.5 min[18]) and quantification limit in saliva to 10 pg/ml (compared to 20 pg/ml[19]). We determined MS/MS optimal analytical conditions using configured IntelliStart software program. Precursor and product ion transitions for the measurement of testosterone and the IS response were set as m/z 289.18 → 97.04 and 292.24 → 97.04, respectively. The MS/MS spectra and chemical structure of testosterone and d3-testosterone are depicted in Figure 1.

## Method validation

We validated the method as previously described[22] and in accordance with published guidelines.[23] Validation included specificity, recovery, precision, accuracy, and stability.

### Specificity

We studied assay specificity using six batches of blank human plasma; none showed interference. Further, the potential interference of structurally similar compounds, i.e., progesterone, 17 α-hydroxyprogesterone, prednisone, prednisolone, methyl prednisolone, and cortisol (1.0 μg/ml in methanol: water, 1:1, v: v) was also examined. No compound showed interference with testosterone or the IS peaks.
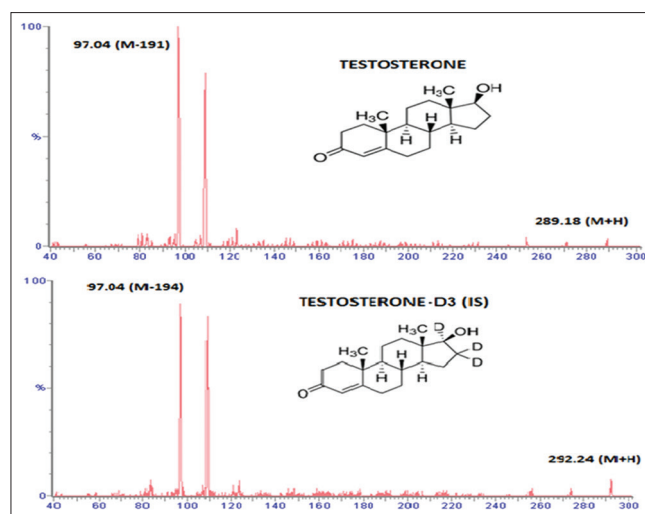


**Figure 1:** Mass spectrometry/mass spectrometry spectra and chemical structures of testosterone and d3-testosterone (internal standard)

Representative chromatograms of IS-spiked blank human plasma and artificial saliva are depicted in Figure 2.

### Extraction recovery

Recoveries of testosterone and the IS from plasma and saliva were assessed using five replicates of four testosterone concentrations and one concentration of the IS. The extraction recovery was in the 92%–99% range for testosterone from plasma, 94%–99% for testosterone from saliva, and 89% and 96% for the IS from plasma and saliva, respectively.

### Linearity and quantification limit

Linearity was evaluated by analyzing testosterone standards at eight concentrations, ranging from 0.5 to 50 ng/ml in plasma and from 10 to 400 pg/ml in saliva. The mean equations ($n = 8$) were $Y = 0.6211 (x) - 0.1808 (r^2 = 0.9975 [0.0022])$ and $y = 0.0048 (x) + 0.0014 (r^2 = 0.9958 [0.0033])$ for plasma and saliva, respectively. The detection and quantification limits were 0.3 ng/ml and 0.5 ng/ml for plasma and 5 pg/ml and 10 pg/ml for salivary samples, respectively.

### Accuracy and precision

We assessed the accuracy and precision of the plasma and salivary assays by measuring testosterone levels at four concentrations in ten replicates [Table 1]. Intrarun imprecision and bias were ≤5.5% and ≤±12.1% and ≤8.7% and ≤±3.8% for plasma and salivary samples, respectively. The corresponding interrun values (determined over 3 days) were ≤12.6% and ≤±7.5% and ≤10.2% and ≤±5.3%, for plasma and salivary samples, respectively.

### Stability

The stability of testosterone and the IS in processed and unprocessed plasma (1.5 and 90 ng/ml) and salivary

**Table 1: Intra- and interrun precision and bias of testosterone assay**

| Nominal level (ng/ml) | Measured level | | | | | |
|---|---|---|---|---|---|---|
| | Intrarun (n=10) | | | Interrun (n=20) | | |
| | Mean±SD | CV (%) | Bias (%) | Mean±SD | CV (%) | Bias (%) |
| Plasma | | | | | | |
| 0.5 | 0.56±0.31 | 5.5 | 12.1 | 0.51±0.06 | 12.6 | 1.3 |
| 1.5 | 1.60±0.04 | 2.2 | 6.9 | 1.56±0.11 | 7.5 | 4.1 |
| 25 | 23.31±1.10 | 4.8 | −6.8 | 23.89±1.22 | 5.1 | −4.4 |
| 45 | 41.07±1.91 | 4.7 | −8.7 | 41.63±1.86 | 4.5 | −7.5 |
| Saliva | | | | | | |
| 10 | 10.1±0.7 | 7.1 | 0.5 | 9.8±0.9 | 10.2 | −2.4 |
| 30 | 29.5±2.6 | 8.7 | −1.7 | 29.3±2.3 | 8.0 | −2.4 |
| 180 | 173.1±12.4 | 7.1 | −3.8 | 170.5±10.7 | 6.3 | −5.3 |
| 360 | 353.9±16.3 | 4.6 | −1.7 | 347.7±18.3 | 5.2 | −3.4 |

CV is calculated as SD/mean measured level time 100. Bias is calculated as measured level minus nominal level/nominal level time 100. SD: Standard deviation, CV: Coefficient of variation
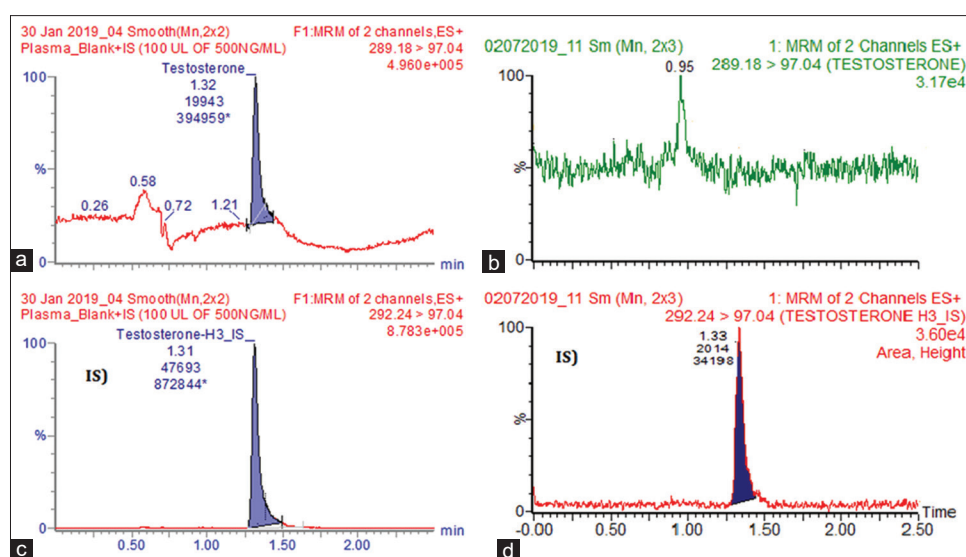


**Figure 2:** Multireaction monitoring chromatogram of blank human plasma (a), artificial saliva (b), IS-spiked human plasma (c) and IS-spiked artificial saliva (d)

(30 and 360 pg/ml) samples was investigated. In both types of samples, testosterone was stable for 24 h at room temperature (≥97%) or 48 h at −20°C (≥93%) in processed samples. In unprocessed samples, it was stable for 24 h at room temperature (≥97%), 8 weeks at −20°C (≥91%), or after three freeze-and-thaw cycles (≥82%).

## CONCLUSIONS

A simple, sensitive, specific, and rapid UPLC-MS/MS assay for measuring clinically relevant testosterone level in human plasma and saliva was developed and validated. The assay requires 0.5-ml human plasma or 1.0-ml artificial salivary samples. It was successfully used to monitor the stability of testosterone and is suitable to investigate testosterone level in clinical practice and clinical research.

### Financial support and sponsorship
Nil.

### Conflicts of interest
There are no conflicts of interest.

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