Journal of
Bioequivalence & Bioavailability
ISSN: 0975-0851

Alvi et al., J Bioequiv Availab 2013, 5:6
DOI: 10.4172/jbb.1000163

Research Article Open Access

Development and Validation of LC-MS/MS Method for Determination of Testosterone Level in Human Saliva Using Lovastatin as Internal Standard

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Abstract

A rapid Liquid Chromatographic Tandem Mass Spectrometric (LC-MS/MS) assay for the measurement of testosterone level in human saliva was developed and validated using lovastatin as internal standard (IS). Retention times of testosterone and IS were around 3.2 and 3.8 min, respectively. Relationship between testosterone concentration and peak height ratio of testosterone to IS was linear ($R^2 \geq 0.98$) in the range of 20-400 pg/ml, and the intra- and inter-day coefficient of variations (CV) were 4.7% to 9.5% and 6.5% to 11.3%, respectively. Quantification and detection limits were 20 and 10 pg/ml, respectively. Mean extraction recoveries were 80-93% for testosterone (four levels) and 81% for the IS. Stability of testosterone was determined under various conditions generally encountered in the clinical laboratory. The method was also successfully applied to determine testosterone level in saliva obtained from healthy subjects.

Keywords: Testosterone; Lovastatin; Saliva; LC-MS/MS

Introduction

Testosterone is an important steroid androgen hormone; its measurement in human plasma/serum has been traditionally used to diagnose and monitor androgen disorders in men and women [1-3].

In recent years, the use of saliva as a diagnostic fluid has gained much interest because of easier sample collection/handling and ability to accurately measure free//bioavailable levels of some hormones [4-8].

Several methods, including radioimmunoassay, enzyme immunoassay, gas chromatography-mass spectrometry, and liquid chromatography- mass spectrometry have been developed for the determination of testosterone level in human plasma/serum [9-14]. Immunoassays, in general, lack specificity, accuracy, and sensitivity [15,16]. Few LC-MS/MS methods have been reported [17,18] for measuring testosterone levels in saliva. However, most use as internal standard deuterated testosterone which is not readily available due to regulatory restrictions.

The objective of the current study was to develop, validate, and apply a simple LC-MS/MS assay for quantification of testosterone level in human saliva using a readily available internal standard. In addition, the study addressed the stability of testosterone under various clinical laboratory conditions.

Materials and Methods

Apparatus

LC-MS/MS analysis was performed on Waters Alliance HPLC 2695 Separation module consisting of quaternary pump, autosampler, column thermostat, and Micromass Quattro micro API bench-top triple quadruple mass spectrometer, interfaced with a Z-spray ESI (Waters Associates Inc, Milford, MA, USA).

Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. Testosterone and Lovastatin were purchased from Sigma Chemical Co., USA and UPS reference standard Rockville, MD, USA, respectively. Ammonium acetate, *tert.* butyl methyl ether, and methanol (HPLC grade) were purchased from Fisher Scientific, NJ. USA. Water for HPLC was prepared by reverse osmosis and further purified by using synergy water purification system (Millipore, Bedford, MA, USA). The study was approved by the Research Ethics Committee, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia.

Chromatographic conditions

Analysis was performed on a reversed phase Atlantis dC18 (2.1×100 mm, 3 µm) column equipped with Symmetry C_{18} (3.9×20 mm, 5 µm) guard column. The mobile phase containing 0.02M ammonium acetate and methanol (90:10, v:v) was filtered through a 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA), degassed, and delivered at a flow rate of 0.2 ml/min. Mass Lynx software (Ver 4.0, Micromass) working under Microsoft Window XP professional environment was used to control the instruments, data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements. The electrospray ionization source was operated in the positive-ion mode at a capillary voltage of 4.0 kV and a cone voltage of 27 V. Nitrogen was used as nebulizing and desolvation gas at a flow rate of 60 and 600 L/hr, respectively. Argon was used as the collision gas at a pressure of 1.28×10^{-3} mbar. The optimum collision energy for testosterone and lovastatin (internal standard, IS) was 27 eV. The ion source and the desolvation temperatures were maintained at 120 and 350°C, respectively.

Standards and controls

Testosterone and IS stock solutions were prepared in methanol

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Received July 05, 2013; **Accepted** September 30, 2013; **Published** October 09, 2013

Citation: Alvi SN, Dgither SA, Hammami MM (2013) Development and Validation of LC-MS/MS Method for Determination of Testosterone Level in Human Saliva Using Lovastatin as Internal Standard. J Bioequiv Availab 5: 228-232. doi:10.4172/jbb.1000163

(100 µg/ml). Calibration standards at nine different concentrations (20-400 pg/ml) and quality controls at four concentrations: 20, 60, 200, and 380 pg/ml were prepared in human saliva. IS working solution was prepared in methanol (10 ng/ml). Standard and control solutions were vortexed for one minute, and 1.0 ml aliquots were transferred into 7 ml glass culture tubes and stored at -20°C until used.

Sample preparation

100 µl of the IS working solution was added to each 1.0 saliva sample, calibration standard, or quality control samples in a 7 mL culture tubes and vortexed. 4.0 ml *tert*. butyl methyl ether was added to each tube then vortexed for one minute and centrifuged at 3000 rpm for 10 minutes at room temperature. The clear supernatant layer was transferred to a clean culture tube and dried under gentle steam of nitrogen at 40°C. The residue was reconstituted in 100 µl solution containing 0.02M ammonium acetate and methanol (1:1, v:v). 10 µl of the clear solution was injected into the LC-MS/MS system.

Calculation

We first calculated the peak height ratios (testosterone to IS) for each standard solution. In order to correct for endogenous testosterone level in blank unspiked saliva, we subtracted the peak height ratio of blank saliva from the peak height ratios obtained using standard solutions. The differences were plotted against the spiked concentration. Bias (%) was calculated as the difference between measured and nominal concentration divided by nominal level times 100, whereas coefficient of variation was calculated as the standard deviation divided by mean concentration times 100.

Results and Discussion

Method development

Mass spectra: Precursor ions of testosterone and IS and their corresponding product ions were determined from spectra obtained during the infusion of standard solutions into the mass spectrometer. Testosterone and IS mainly produced m/z 289.1 and 427, respectively. The product ion transitions were quantitatively measured as peak height at m/z 289.1 → 97 for testosterone and 427.3 → 325 for IS in multiple reaction mode. Chemical structures and MS-MS spectra of testosterone and IS are depicts in Figure 1.

LCMS/MS conditions optimization: Detection and quantification of testosterone were optimized using a mobile phase composed of 0.02M ammonium acetate and methanol (90:10, v:v) and a flow rate 0.2 ml/min. The high proportion of ammonium acetate in the mobile phase allowed completing the analysis in 5.0 minutes. Figure 2 depicts

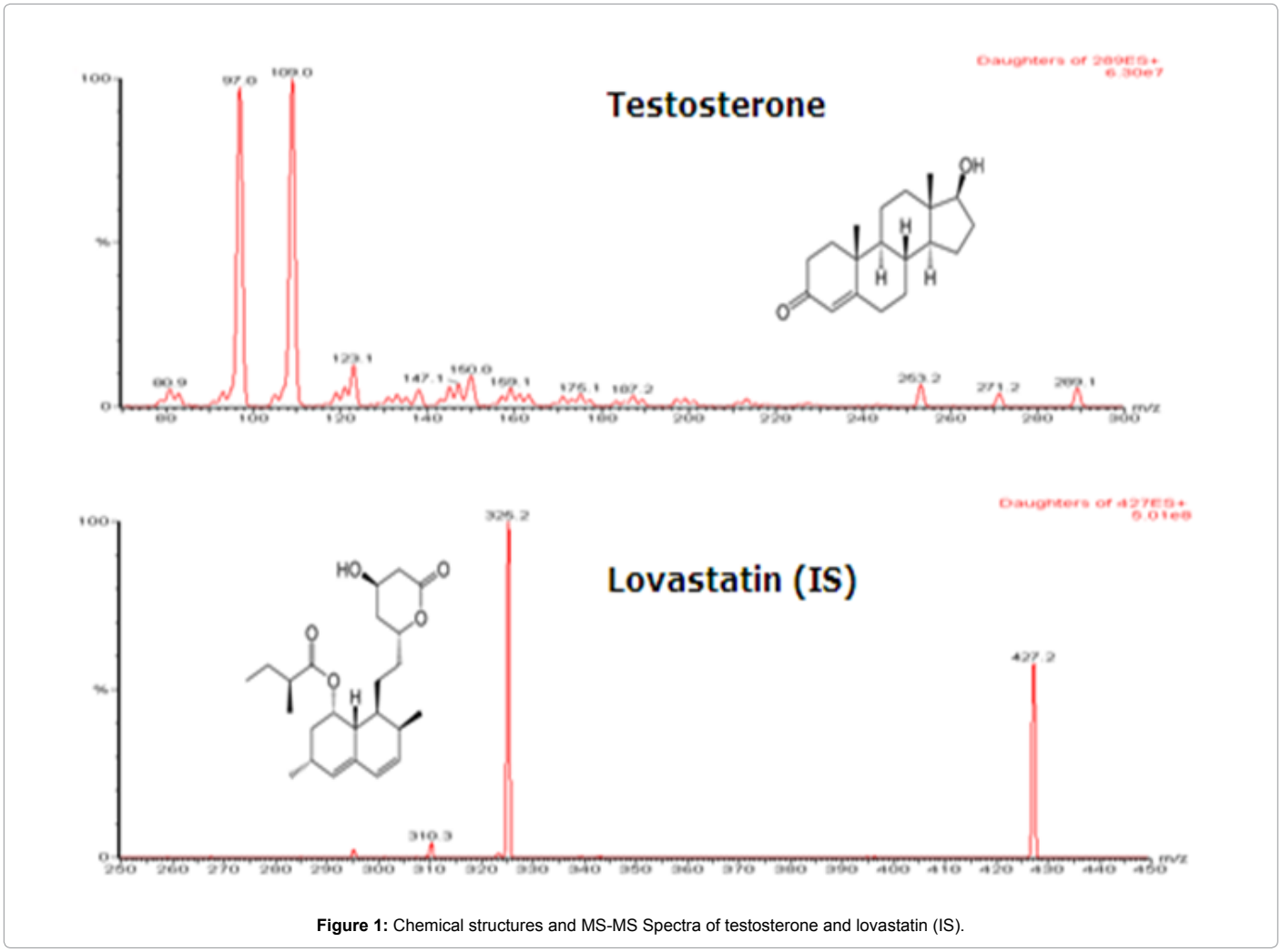

Figure 1: Chemical structures and MS-MS Spectra of testosterone and lovastatin (IS).

representative chromatograms of saliva spiked with 10 ng/ml IS and testosterone at four concentrations (20, 60, 200 and 360 pg/ml).

Extraction recovery: The absolute recovery of testosterone was assessed by comparing absolute peak heights of spiked saliva and mobile phase samples, using five replicates for each of four concentrations (20, 60, 200 and 360 pg/ml). Similarly, the recovery of the IS was determined by comparing the peak height of the IS in 5 aliquots of human saliva spiked with 10 ng/ml IS with the peak height of equivalent samples prepared in mobile phase. Mean extraction recoveries were 80-93% for testosterone and 81% for the IS, The results are presented in Table 1.

Method validation

The procedures used for validation were according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance [19].

Specificity: To evaluate assay specificity, we screened six different batches of human plasma and eight frequently used medications namely: acetaminophen, ibuprofen, aspirin, ranitidine, nicotinic acid, ascorbic acid, caffeine, and omeprazole for potential interference. None was found to interfere with the quantification of testosterone or the IS.

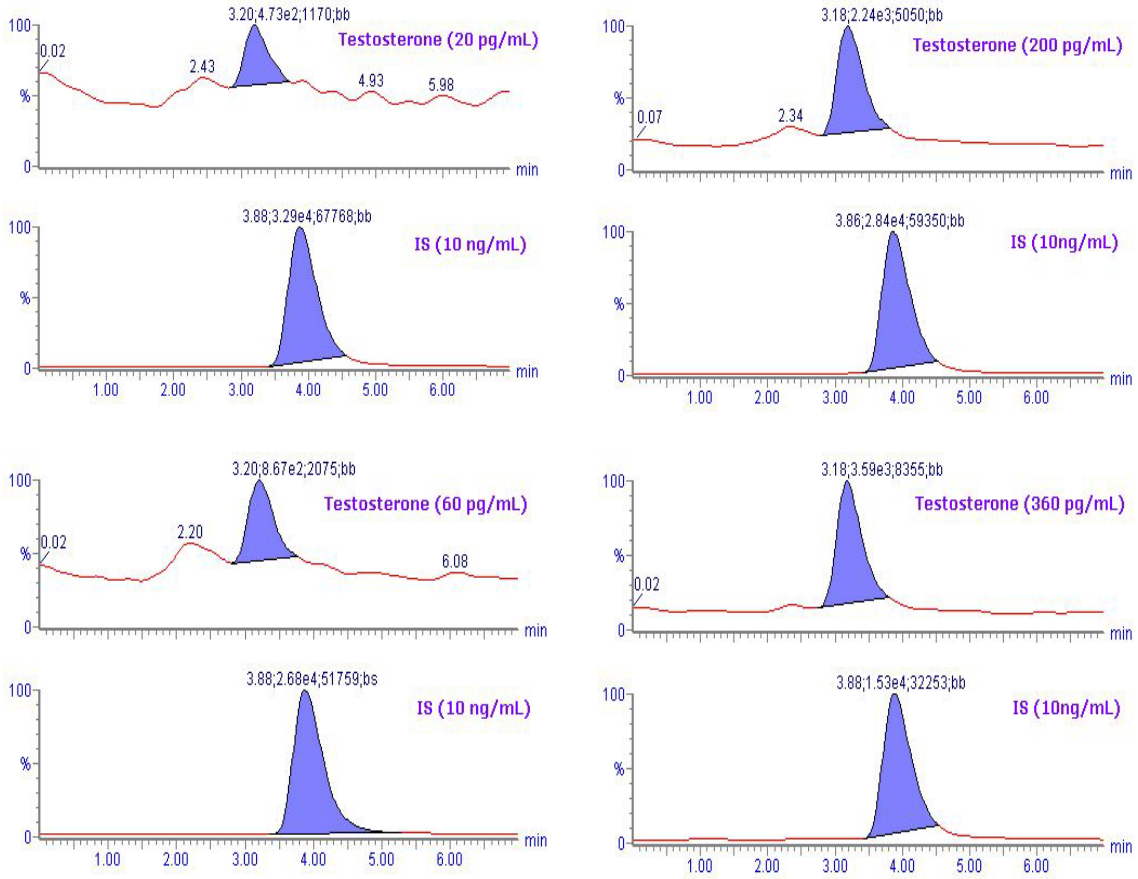

Figure 2: Quality control samples of human saliva spiked with testosterone at four concentrations (20, 60, 200 and 360 pg/ml).

Testosterone (pg/mL)	Human Saliva		Mobile Phase		†Recovery (%)
	*Mean Peak Height	SD	*Mean Peak Height	SD	
20	496	68	611	69	81
60	1532	151	1840	152	83
200	3920	208	4925	277	80
360	8267	327	8920	748	93
IS (10 ng/mL)	9384	180	11570	225	81

*Mean of 5 replicate. †Mean peak height of testosterone in saliva divided by mean peak height in mobile phase X 100, SD, Standard deviation

Table 1: Extraction recovery of testosterone and Lovastatin (IS).

Linearity and limit of quantification: Linearity of the assay was evaluated by analyzing a series of standards at nine different concentrations over the range of 20 - 400 pg/ml. Corresponding peak height ratios and concentrations were subjected to regression analysis. The mean equation obtained from six standard curves was y= -0.0004 x - 0.0003, R^2=0.98 (±0.0034). Quantification limit was established as 20 pg/ml and detection limit as 10 pg/ml.

Accuracy and Precision: Accuracy and precision were determined by measuring levels of testosterone in four spiked saliva samples (20, 60, 200, 380 pg/ml). The intra-and inter-day imprecision and bias were determined over three different days. Intra-day (n=10) imprecision and bias were ≤ 9.5% and ≤ 8.7%, respectively. Inter-day (n=20) imprecision and bias were ≤ 11.3 % and ≤ 4.7%, respectively (Table 2).

Robustness: The robustness of the current assay was evaluated by altering the strength of ammonium acetate (± 0.02%), and the amount of methanol (± 2.0 %) in the mobile phase. No significant effects were observed.

Nominal Level (pg/ml)	Intra-day *Measured Levels				Inter-day *Measured Levels			
	Mean	SD	CV (%)	Bias (%)	Mean	SD	CV (%)	Bias (%)
20	19.4	1.8	9.5	3.0	20.2	2.3	11.3	1.0
60	57.3	3.3	5.7	4.5	57.2	3.7	6.5	4.7
200	210.1	17.2	8.2	5.1	202.6	21.0	10.4	1.3
360	391.3	18.5	4.7	8.7	373.1	30.2	8.1	3.6

SD, standard deviation, CV, Coefficient of variation as a measurement of imprecision= standard deviation divided by mean measured concentration x 100. Bias, measured level - nominal level divided by nominal level x 100

Table 2: Intra - and inter-run precision and accuracy of testosterone assay.

Nominal Level (pg/ml)	Stability (%)								
	*Saliva samples				**Stock Solution				
	Unprocessed		Processed		Freeze –Thaw Cycle				
	24 hrs	8 weeks	24 hrs	48 hrs				24hrs	8wks
	RT	-20°C	RT	-20°C	1	2	3	RT	-20 °C
60	105	105	94	102	102	103	102		
200	106	111	107	92	95	97	91	102	111
360	101	99	102	94	90	89	94		

Stability (%)=mean measured concentration (n=5) at the indicated time divided by the mean measured concentration (n=5) at baseline x 100. *Spiked saliva samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), after freezing at –20ºC for 8 weeks (8 wks –20ºC), or after 1 to 3 cycles of freezing at –20ºC and thawing at room temperature (freeze-thaw), or processed and then analyzed after storing for 24 hours at room temperature (24 hrs RT) or 48 hours at –2ºC (48 hrs -20ºC).** Stock solution, 100 µg/mL in methanol

Table 3: Stability of testosterone in different conditions.

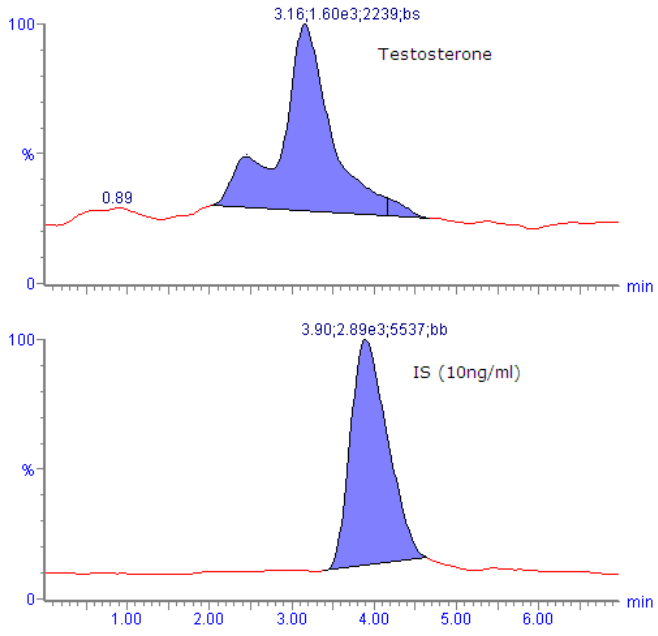

Figure 3: Representative chromatogram of a volunteer's saliva sample.

Stability

Stability of the testosterone in unprocessed and processed saliva samples was investigated under various conditions. Stock/working solutions, saliva samples, processed samples were studied after being maintained at room temperature for up to 24 hours. In addition, saliva samples containing testosterone (60 and 360 pg/ml) were stored at -20°C for eight weeks and were exposed to three freeze-thaw cycles. No decrease in the measured concentration or change in chromatographic behavior of testosterone or IS was observed. Table 3 summarizes the results of stability studies.

Application to Volunteer Samples

Approximately 4.0 ml saliva was collected in duplicate from each volunteer in clean polypropylene centrifuge tube by direct spitting in the tube and stored at -20°C until analyzed. Over 100 saliva samples were analyzed by the method without significant loss of resolution, change in column efficiency, or increase in back pressure. Figure 3 depicts representative chromatograms of saliva samples obtained from a healthy volunteer. The measured level of testosterone was 165.7 pg/ml.

Conclusions

The described LC-MS/MS method is simple, precise, and accurate for rapid measurement of testosterone level in human saliva. The assay was applied to monitor testosterone stability under various condition encountered in the clinical laboratory. Further, the assay was successfully applied to determine testosterone level in saliva samples obtained from healthy volunteers.

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