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Analytical Method Validation of Testosterone Undecanoate Soft Gelatin Capsule by RP-HPLC Method

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Abstract

A rapid, sensitive, selective reversed phase HPLC method has been validated for the quantification of testosterone undecanoate from Andriol® soft gelatin capsule. During validation active pharmaceutical ingredient (API) has been separated by C18 (4.6 mm × 250 mm, 5 µm) column, 100% methanol as mobile phase, flow rate of 0.8 ml/min and detection wavelength at 240 nm. The method was validated according to USP and ICH guideline requirements which includes specificity, accuracy, precision, linearity and range and robustness. Linearity of standard spiked sample was observed for each working day and coefficient of determination (r^2) has been found >0.99 each day in concentration ranging from 20-60 ppm. Recovery was found from 98.87-100.02% for 20, 40 and 60 ppm of testosterone undecanoate spiked sample. Precision and intermediate precision showed that % RSD of test sample solution were 0.26 and 0.19 respectively and absolute difference between them was 0.52, all of the values were within acceptable limit. The method was also found robust in changing column oven temperature (± 5°C) and flow rate change (± 0.1).

Keywords: Chromatography; Linearity; Precision; Accuracy; Calibration

Introduction

Testosterone is a principal hormone Responsible for the formation and maintenance of libido, sexual interest and sexual activity in men [1,2]. In addition, it is important for non-reproductive tissues, such as muscle, bone, hair follicle, larynx, skin, adipose tissue, kidney and brain functioning. 95% of Testosterone is secreted from the leydig cells of testes and produce 5 to 10 mg/day. Testosterone is mainly is bound with albumin protein with low affinity and to sex hormone binding globulin (40-50%) with high affinity. 1 to 2% of it is not bound with protein and represents the free state and considered the biologically active testosterone and available for tissue uptake [2]. Testosterone value in serum greater than 12 nmole/L is normal but less than 8 nmole/L is considered hypogonadal and testosterone replacement is commensurate [3]. Low levels of it in human body may create several high-risk factors such as metabolic syndrome [4,5], obesity [6], type 2 diabetes mellitus (T2DM) [7,8], atherosclerosis [9], chronic heart failure [10], cardiovascular disease [11] and erectile dysfunction (ED) etc. [12].

There are several routes of testosterone administration in human body such as intramuscular injection, wax pellets that are inserted into a deep subcutaneous, oral tablet, gels, patches and capsule by Testosterone and Testosterone ester [2,3,13,14]. Testosterone, administered through intramuscular injection, skin patch and wax pellets that are inserted into a deep subcutaneous for androgen supplement therapy has been used for the treatment for hypogonadism or andropause [15-19]. Though those therapies have an appropriate endocrine effect, they have some disadvantage like inability to maintain serum concentration, adverse reactions and cost effectiveness [14,20]. But an alternative oral dosage form used for hypogonadism may be the non-alkylated testosterone ester and testosterone undecanoate that can deliver testosterone to the systemic circulation via the intestinal lymphatic route with very little side effect [20]. Subdermal testosterone wax pellets require minor surgery for insertion and often cause local problems [14].

Oral administration of pure and crystalline testosterone metabolized in intestinal well and 98% of total amount has been absorbed and remain inactivated. So pure and crystalline form of oral administration of testosterone undecanoate does not increase serum testosterone in sufficient levels and not suitable for hypogonadal disorder treatment [21]. For that reason, oral testosterone undecanoate solution in oily vehicle, contained in a soft gelatin capsules has been invented. Despite other capsule gives better pharmacokinetics profile, improve hypogonadal men report symptoms and long-term safety data [22,23].

Many analytical methods has been proposed for quantitative determination of testosterone undecanoate such as UV method [24,25], gas chromatography-mass spectroscopy [21,26,27], LC-MS/MS method [28-31], nuclear magnetic resonance [32], LC-Q-TOF/MS [33].

The recent study is based on validating a rapid, sensitive and selective reversed phase HPLC method to quantify testosterone

undecanoate accurately and precisely from Andriol® soft gelatin capsule according to ICH and USP guideline [34,35] (Figure 1).

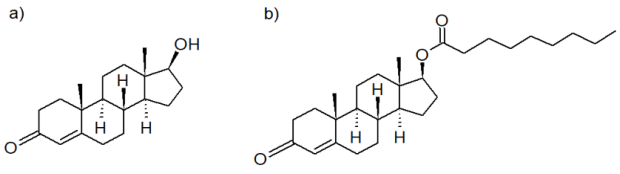

Figure 1: Structure of a) Testosterone b) Testosterone Undecanoate.

Materials and Methods

Materials

Testosterone Undecanoate certified reference standard was purchased from the Excella GmbH & Co (Germany). Andriol soft gelatin capsule was purchased from local market, Chromafil® Xtra PTFE 0.45 µm syringe filters were purchased from the Pall Corporation (Ann Arbor, MI, USA). HPLC grade methanol was purchased from Fisher Scientific (Fairlawn, NJ, USA). HPLC ready deionized 18Milli-Q water was obtained, in-house, from a Milli-Q Gradient A-10 water purification system, Millipore, (Bedford, MA, USA).

Calculation

Testosterone undecanoate in test sample was calculated in quantitative and percentage basis from measured peak area response for the test sample (A_u), compared to standard peak area response (A_s) using following equations:

$$\text{Quantity} = A_u/A_s \times C \quad \ldots\ldots\ldots\ldots\ldots (1)$$

$$\% \text{ Recovery} = (\text{Observed Amount})/(\text{Declared Amount}) \times 100 \quad \ldots\ldots\ldots (2)$$

Where C is the concentration in ppm of the Testosterone Undecanoate.

Instrumentation and chromatographic conditions

Prominence I HPLC (Shimadzu Corporation, Japan) consisted of a quaternary pump, an automatic injector, variable wavelength detector, and a column oven was used for analysis. Data were processed by using Lab solution 6.82-ST1 software. Chromatographic separation of testosterone were performed using Agilent C_{18} (4.6 mm × 250 mm, 5 µm) and ProntoSIL columns, column oven temperature of 25°C and eluted with mobile phase flow rate of 0.8 ml/min. The mobile phase was only 100% methanol which was filtered 0.45 µm nylon filter and degassed in ultrasonic bath before use. Measurement were done with injection volume of 10 µl and detector wavelength at 240 nm.

Stock solution preparation (400 ppm)

20 mg of testosterone undecanoate chemical standard was transferred in 50 ml volumetric flask dissolved it with methanol with proper sonication.

Standard solution preparation (40 ppm)

2 ml of stock solution were transferred in to 20 ml volumetric flask and volume to the mark with diluent.

Preparation of calibration standard solution

Testosterone Undecanoate stock solution were used to prepare calibration standard solution in daily basis. It was prepared by using 5 concentrations with three replicates by diluting stock solution to the concentrations of 20, 32, 40, 48 and 60 ppm. Those solutions were then transferred in HPLC vial for analysis.

Test stock solution preparation (400 ppm)

350 mg (equivalent of 40 mg Testosterone Undecanoate) of test sample was taken in a 100 ml volumetric flask. Dissolve it with proper sonication and then volume to the mark with diluent.

Test solution preparation (40 ppm)

5 ml of above stock solution was then transferred in 50 ml volumetric flask and volume to the mark with same diluent.

Results and Discussion

Method validation

The method was validated according to the ICH and United States Pharmacopeia Category I requirements [34,35]. The following validation characteristics were addressed: specificity, accuracy, precision, linearity and range and robustness.

System suitability standard

System suitability solution was prepared from daily using stock solution, for that purpose 2 ml stock solution was transferred to 20 ml volumetric flask and volume to the mark with diluent. System suitability was determined by injecting five replicate standard solution from same vial before analyze test sample each day. According to USP and ICH guideline the acceptance criteria for system suitability were: relative standard deviation should be less than 2, theoretical plates should be greater than 2000 and tailing factor should be less than 2 [34,35]. During analysis it has been found that all parameter met the acceptable criteria throughout all days which is shown in the Table 1 (Figure 2).

Specificity

Specificity of an analytical method means to show that the method was not affected by the presence of impurities or excipients or and with diluent. The acceptance criteria is peak of active in test sample should be pure that means diluent and placebo does not show any interfere at the retention time of active components. It was found from the chromatogram that there were no interference at 11.6 min retention time of Testosterone Undecanoate, whereas diluent peak was found at 3.5 min and placebo peaks were found at 5.85, 7, 7.7, 9.8, 12.4 and 13.5 min. All of the peaks of diluent, placebo and Testosterone Undecanoate were shown in the Figure 3 with data comparison.

Parameter	Specifications	Day 01	Day 02	Day 03	Day 04
Retention Time (% RSD)	≤ 2.0	0.02	0.02	0.01	0.0
Area (% RSD)	≤ 2.0	0.17	0.67	0.22	0.19
Tailing Factor	≤ 2.0	1.02	0.97	0.94	0.96
Theoretical plates	≥ 2000	9780 ± 103	11286 ± 67	8882 ± 75	11897 ± 156

Table 1: System suitability test results (n=5), n: number of replicates per concentration levels and per series.

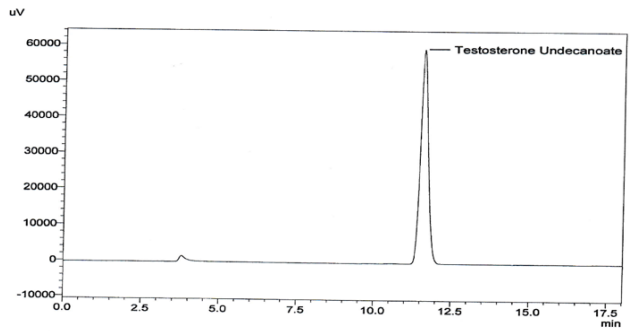

Figure 2: Standard peak of Testosterone Undecanoate.

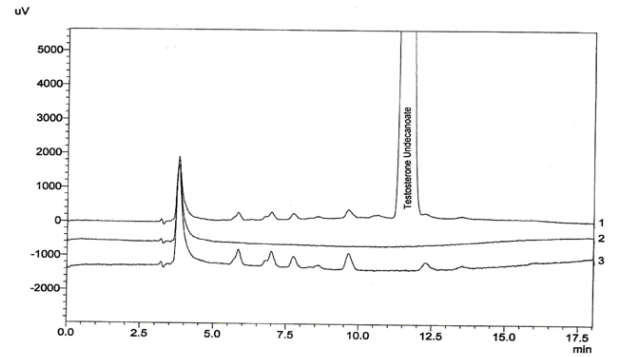

Figure 3: Data comparison among diluent, placebo and test sample (Andriol® soft gelatin Capsule) 1: Test Sample, 2: Diluent and 3: Placebo.

Precision and intermediate precision

Precision expressed as an absolute or relative standard deviation (RSD) and does not relate to reference values or actual value. On the other side intermediate precision expressed to determination of RSD of replicate sample within laboratories variations: different days, different analysts, different column, different HPLC etc. [36].

Precision and intermediate precision solution were prepared from stock solution same as system suitability solution preparation and concentration was 40 ppm. Precision and intermediate precision test were done by injecting six replicate standard solution. Results for precision and intermediate precision were summarized in Table 2.

Parameter	Specifications	Precision	Intermediate Precision
Area of Sample	-	1008773 ± 0.17	1038367 ± 0.67
Amount Recovered	95-100%	101.12	101.64
Recovery (% RSD)	≤ 5.0	0.26	0.19
Absolute difference	<2.0	0.52	

Table 2: Precision and intermediate precision Results (n=6), n: number of replicates per concentration levels and per series.

Accuracy

Accuracy expresses the closeness of agreement between the measured value and the value that is accepted as either a true value or a reference value [37]. Accuracy of this method assessed by analyzing three different known concentrations (20, 40 and 60 ppm) that were prepared from test stock solution and compared the measured value with true value.

According to USP guideline accuracy of assay samples should be within 98.0 to 102.0% [38]. From the analysis recovery of Testosterone Undecanoate was found from 98.87 to 100.02% for three concentration levels which is summarized in the Table 3.

Parameter	Specifications	Testosterone Undecanoate		
		20 ppm	40 ppm	60 ppm
Recovery (%)	98.0-102.0	100.02 ± 0.54	98.87 ± 0.11	99.19 ± 0.04
Recovery (mg)	-	20.00 ± 0.11	39.55 ± 0.04	59.52 ± 0.02

Area	-	516943 ± 0.46	1016845 ± 0.05	1530408 ± 0.08

Table 3: Accuracy Results (n=6), n: number of replicates per concentration levels and per series.

Linearity and range

Linearity is the ability of a method to test the relationship between analysts concentration with its response (area). According to USP, IUPAC, ICH and some literature for assay linearity test should be done from 80 to 120% of the target concentration with 5-8 concentration levels and 2-6 replicates should be analyzed per concentration and within that range coefficient of determination (r^2) should be greater than 0.99 [34,35,39,40]. For linearity standard calibration curves were prepared with five calibrators over a concentration range from 20 to 60 ppm with 3 replicates per concentration. Correlation between analyte peak area and concentration were estimated and it was observed that coefficient of determination were >0.99 for all days throughout the analysis which is shown in the Table 4.

Standard Curve	Analytical Range (ppm)	Slope	y-intercept	r^2 value
Validation day 1	20-60	27600	2596.5	0.9999
Validation day 2	20-60	27514	32519	0.9998
Validation day 3	20-60	27179	-4927	0.9984
Validation day 4	20-60	26598	-45218	0.9995

Table 4: Linearity results (m=5; n=3), m: number of concentration levels or calibrator; n: number of replicates per concentration levels and per series.

Robustness

Robustness of an analytical procedures has been defined by the International Conference on Harmonization (ICH) as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters [41]. For the determination of a methods robustness, many method parameters, such as pH, flow rate, column temperature, column oven temperature and column variation etc. [42]. If the influence of the parameter was within acceptance range, the parameter was said to be robust.

Robustness of the method was carried out by deliberately making variation in the flow rate (± 0.1 ml/min.) and changing column oven temperature (± 5°C). During performing robustness test standard stock solution at concentration of 40 ppm Testosterone Undecanoate was used and it was found that all the criteria for system suitability was satisfactory. So that it can be concluded that this method was robust at that changing parameter. The results is summarized in the Table 5.

Parameter	Value	Retention time	Tailing factor	Theoretical plate	Area of Standard	Recovery (%)
Acceptance Criteria	-	-	≤ 2.0	≥ 2000	-	98.0-102.0
Control	As per method	11.70	1.02	9780	112645	100.14
Flow rate (ml/min)	0.7	14.11	1.05	11870	138366	99.05
	0.9	11.23	1.24	8457	109239	101.49
Column oven temperature (°C)	20	14.72	0.96	9402	132299	100.84
	30	12.95	1.03	9140	135506	100.44

Table 5: Robustness results (n=3), n: number of replicates per concentration levels and per series.

Force degradation

To force degradation ICH recommends conducting stress studies, in conditions such as elevated temperature, humidity, acidic, basic, oxidation and light to demonstrate the specificity of the assay in presence of degradation products. According to ICH guideline for drug substance variety of stress condition should be performed with degradation up to about 5-20% [43].

For thermal degradation 350 mg of test sample was transferred in a 100 ml volumetric flask, kept in hot oven at 105°C for 48 hours. Cooled the solution at room temperature and volume to the mark with diluent. Collect 5 ml above solution in 50 ml volumetric flask dilute with diluent. For acid and alkali hydrolysis samples were treated with 10 ml of 1 M HCl and 1 M NaOH and then sonicate for 30 min and then stayed for 1.5 hours. Neutralize the sample solution with 10 ml of base and acid then volume to the mark with diluent. Dilute 5 ml of above solution to 50 ml volumetric flask. Oxidation degradation sample was

prepared by taking 350 mg of test sample in 100 ml volumetric flask and then add 10 ml 10% H_2O_2 with sonication for 60 min which was followed by heating in water bath at 60°C for 2 hours. Cooled the solution at room temperature and then volume to the mark. Dilute 5 ml of above solution in 50 ml volumetric flask (Table 6).

Stress Condition	Area of API before degradation	Area of API after degradation	% Degradation
Thermal	1042257	846625	18.77
Acidic	1042257	916873	12.03
Alkali	1042257	840893	19.32
Oxidation	1042257	855172	17.95

Table 6: Force degradation results (n=3), n: number of replicates per concentration levels and per series.

Conclusion

A simple and effective HPLC method has been validated for assay of Andriol® soft gelatin capsule and successfully determined testosterone undecanoate. The method fulfill all criteria of analytical validation characteristics such as accuracy, precision, specificity, linearity and robustness according to USP and ICH. It can be successfully be used for the analysis of testosterone undecanoate from Andriol® gelatin capsule.

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