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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCES OF TESTOSTERONE UNDECANOATE IN CAPSULE DOSAGE FORM

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ABSTRACT

Simple RS method is developed and validated as reversed-phase chromatographic method for the identification and quantification of the Testosterone undecanoate related substances. The chromatographic separation was optimized with the Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm. A gradient elution was involved with the Acetonitrile and Water (95:5). The flow rate of the mobile phase and the column temperature was set as 1.5 mL min^{-1} and 25°C. The detection wave length was optimized at 240 nm, 20µL Capsule volume. A mixture of Water and Acetonitrile 5:95 v/v was used as diluent, and same diluent used for needle wash purpose also. The method is validated as per the ICH guidelines. There should not be any interference of blank and placebo peaks at the Retention Time of the main analyte and its impurities. Intermediate Precision is 0.8. LOD and LOQ are 0.03 and 0.10 PPM. The correlation coefficient is NLT 0.99. No deliberate change in Peak area with a slight change in Wavelength, Flowrate, Column Temp. & Gradient variationRS method for related substances in Testosterone undecanoate is found specific, linear, accurate, precise, rugged and robust hence the validated method is suitable to identify the related substances in Testosterone undecanoate drug.

KEYWORDS: Rp-Hplc, Method Development, And Validation, Related Substances, Testosterone Undecanoate.

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] IUPAC name is (1S,3aS,3bR,9aR,9bS,11aS)-9a,11a-dimethyl-7-oxo-1H,2H,3H,3aH,3bH,4H, 5H,7H,8H,9H,9aH, 9bH,10H,11H,11aH-cyclopenta[a] phenanthren-1-yl undecanoate. Molecular formula $C_{30}H_{48}O_3$. Molecular Weight is 456.7.

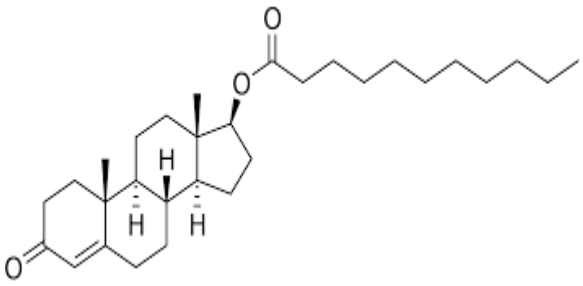

Figure 1: Structure of Testosterone undecanoate.

The literature survey revealed that There are very few methods reported in the literature for analysis of Testosterone undecanoate alone or in combination with other drugs in the pure form and pharmaceuticals formulations.[12-20] In view of the need for a suitable, cost-effective RP-HPLC method for routine analysis of Testosterone undecanoate estimation of in pharmaceutical dosage form. Attempts were made to develop simple, precise, accurate and cost-effective analytical method for the estimation of Testosterone

undecanoate. The proposed method will be validated as per ICH guidelines. The objective of the proposed work is to develop a new, simple, sensitive, accurate and economical analytical method and validation for the estimation of Testosterone undecanoate in pharmaceutical dosage form by using RP-HPLC. To validate the developed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form.

MATERIALS AND METHODS

Chemicals and Reagents: Testosterone undecanoate Gift samples obtained from Symbiotec Pvt. Ltd, Hyderabad. NaH_2PO_4 was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions: The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, UV detector and Empower 2 software. Analysis was carried out at 240 nm with column: Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm, dimensions at Ambient temperature. The optimized mobile phase consists of ACN: Water (95:5 % v/v). Flow rate was maintained at 1.5 ml/min.

Preparation of solutions

Preparation of Testosterone Decanoate impurity stock solution

Weighed accurately 2 mg of Testosterone Decanoate impurity into 100 mL volumetric flask with 30 mL of diluent and sonicated to dissolve the content. Made up the volume with diluent and mixed well.

Resolution solution preparation

Weighed accurately 50 mg of Testosterone Undecanoate working standard into 50 mL volumetric flask. Then 5 mL of Testosterone Decanoate impurity stock solution and 30 mL of diluent were added. Finally, the content was sonicated and made up the volume with diluent.

Standard preparation

25 mg of Testosterone Undecanoate Working standard was weighed accurately in a 100 mL volumetric flask. 30 ml of Acetonitrile was added to it and sonicated for 10 minutes. Then the volume was made up with Acetonitrile. Further diluted 2 ml of the above solution to 100 ml with diluent and mixed well.

Placebo preparation

Weighed accurately 50 mg equivalent of Testosterone Undecanoate placebo into 50 ml volumetric flask. 10 ml of water was added to it & sonicated for 10minutes and then 25mL of Acetonitrile was added & sonicated for 15minutes with intermediate shaking. Volume was made up with diluent. Finally, the placebo solution was centrifuged for about 10 minutes at 2500 rpm and injected into the chromatographic condition.

Sample preparation

20 capsules were taken and weighed to calculate the net content of the capsules. Then weighed 50mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask with 10 ml of water and sonicated for 10minutes. Then 25mL of Acetonitrile was added to it and sonicated for 15minutes with intermediate shaking. Volume was made up with the diluent. Finally, the solution was centrifuged for about 10 minutes at 2500 rpm and injected into the chromatographic condition.

METHOD

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters: To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.5 ml/min for 60 minutes to equilibrate the column at ambient temperature. The overlay spectrum of Testosterone undecanoate was obtained and the Testosterone undecanoate showed absorbance's maxima at 240 nm. Chromatographic separation was achieved by injecting a volume of 20 µL of standard into Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm, the mobile phase of composition ACN: Water (95:5 % v/v) was allowed to flow through the column at a flow rate of 1.5 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Testosterone undecanoate in Capsule dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2.

Validation of Analytical method

Linearity: The linearity study was performed for the concentration of 50 µg/ml to 200 µg/ml level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are shown in table 3.

Accuracy studies: The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150%. Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Testosterone undecanoate and calculate the individual recovery and mean recovery values. The results are shown in table 4.

Precision Studies: precision was calculated from Coefficient of variance for six replicate Capsules of the standard. The standard solution was injected for six times and measured the area for all six Capsules in HPLC. The %RSD for the area of six replicate Capsules was found. The results are shown in table 5.

Ruggedness: To evaluate the intermediate precision of the method, Precision was performed on different day. The standard solution was injected for six times and measured the area for all six Capsules in HPLC. The %RSD for the area of six replicate Capsules was found. The results are shown in table 6.

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition was made to evaluate the impact on the method. The results are shown in table 7.

LOD and LOQ: The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in table 8.

LOD = 3.3σ/S and

LOQ = 10 σ/S, where

σ= Standard deviation of y intercept of regression line,

S = Slope of the calibration curve

Forced Degradation Study

A study was conducted to demonstrate the effective separation of Degradation from Testosterone Undecanoate Soft Gelatin Capsules 40mg of related substances method. Drug product, Placebo, and blank were exposed to the following stress conditions to induce degradation.

Stressed samples of the drug product and Placebo were injected separately into the HPLC system equipped with PDA (Photo Diode Array) detector by using test method conditions.

The results are shown in table 9.

RESULTS AND DISCUSSION

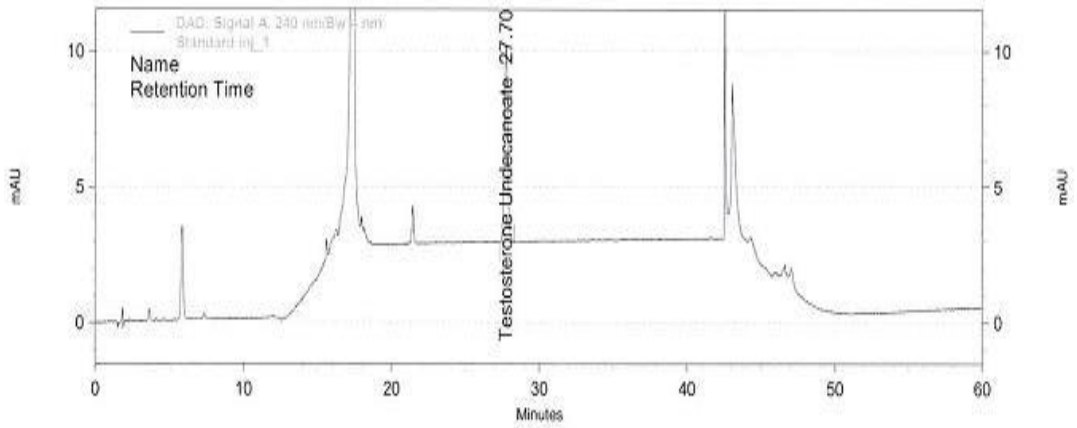

Figure 2: Standard chromatogram.

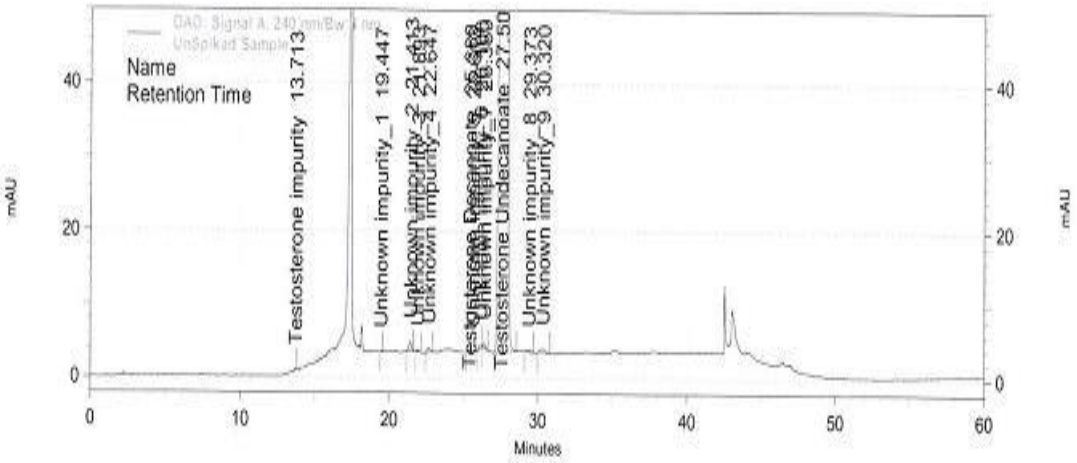

Figure 3: Sample chromatogram.

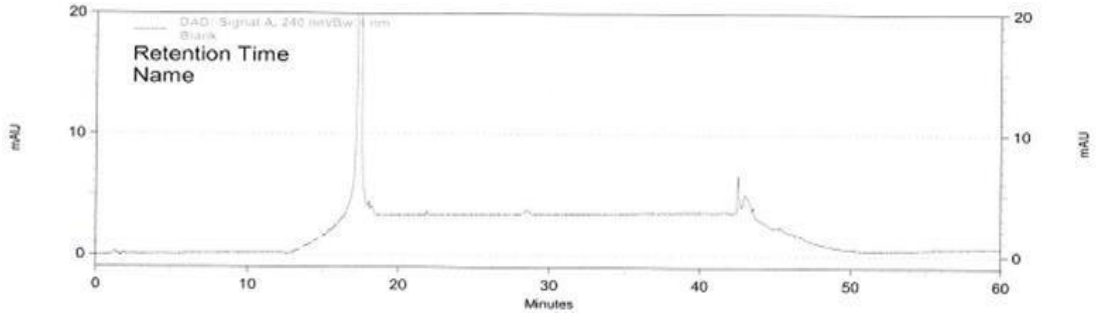

Figure 4: Blank chromatogram.

Table 1: System suitability parameters.

Parameters	%RSD Peak Area	Tailingfactor	Theoreticalplates	Resolution
System Precision	0.2	1.0	65394	5.51
Method Precision	0.2	1.0	65394	5.51
Intermediate Precision	0.1	1.0	60422	5.41
Specificity	0.3	1.0	61207	5.77
Forced degradation-1	0.5	1.0	55242	5.59
Forced degradation-2	0.3	1.2	21733	3.84
Forced degradation-3	0.3	0.8	42039	5.18
LOD and LOQ precision	0.6	1.0	60111	5.82
Linearity	2.8	1.0	63622	5.43
Accuracy	0.2	1.0	69907	5.85
Solution stability (Initial)	0.1	0.9	60103	5.43
Solution Stability (Day-1)	1.9	0.9	47101	4.98
Solution Stability (Day-2)	0.1	1.0	55927	5.20
Mobile phase stability (Initial)	0.8	1.0	64356	5.91
Mobile phase stability (Day-1)	0.4	1.0	62603	5.79
Mobile phase stability (Day-2)	0.2	1.0	63444	5.86
Low Flow Variation (Test condition)	0.3	1.0	61207	5.77
High Flow Variation (Test condition)	0.3	1.0	61207	5.77
High Column Temperature variation	0.6	1.0	60111	5.82
Wavelength variation (Test condition)	0.3	1.0	61207	5.77
Gradient variation-10% (Test condition)	0.2	1.0	60342	5.70
Gradient variation-3% (Test condition)	0.4	1.0	60020	5.77
Filter validation-1	0.2	1.0	65394	5.51
Filter validation-2	0.1	0.9	60103	5.43

Table 2: Summary results of Specificity (Blank and Placebo Interference).

Sample ID	Interference results			
	RT	RRT	Peak Purity	Result
Blank	NAP	NAP	NAP	No interference
Placebo	NAP	NAP	NAP	No interference
Testosterone Undecanoate standard	29.093	1.00	1.0	Nil
Testosterone Decanoate – Resolution solution	26.487	0.91	1.0	Nil
Testosterone Undecanoate – Resolution solution	28.993	1.00	1.0	Nil
Spiked Sample Solution				
Testosterone	13.360	0.46	1.0	Nil
Testosterone Decanoate	26.493	0.91	1.0	Nil
Testosterone Undecanoate	28.993	1.00	1.0	Nil
Unspiked Sample Solution				
Testosterone	13.360	0.46	1.0	Nil
Testosterone Decanoate	26.487	0.91	1.0	Nil
Testosterone Undecanoate	28.993	1.00	1.0	Nil
Individual Impurity				
Testosterone	13.347	NAP	1.0	Nil
Testosterone Decanoate	26.487	NAP	1.0	Nil

Table 3: Linearity results of Testosterone undecanoate.

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone
1	LOQ	0.1003	67615
2	50	5.1487	3504331
3	75	7.7231	5285743
4	100	10.2975	7013181
5	125	12.8719	8620399
6	150	15.4462	10592850
7	200	20.5950	13779847
Slope			671712
Intercept			53992
Coefficient of correlation (r)			0.9998
Coefficient of regression (r^2)			0.9996
% Y-Intercept			0.77
Standard Deviation			106544
LOQ			1.586
LOD			0.523
RRF			1.37

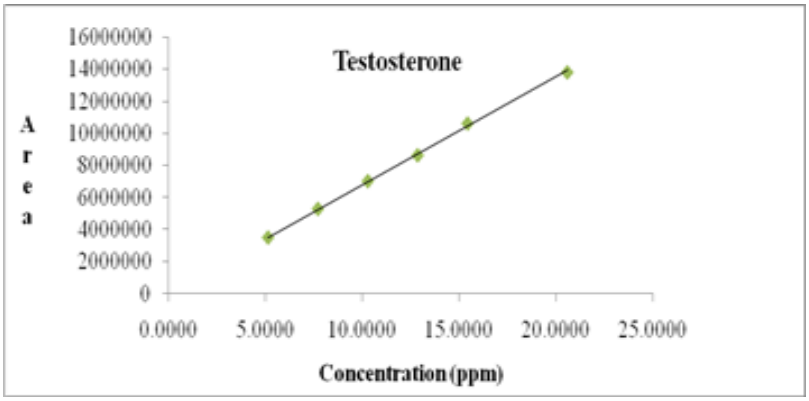

Figure 5: Linearity graph for Testosterone undecanoate.

Table 4: Showing accuracy results for Testosterone undecanoate.

Levels	No of Sample	Added in ppm	Foundin ppm	% Recovery	Average %recovery	%RSD
LOQ	1	0.2000	0.2290	114.5	112.1	4.4
	2	0.2000	0.2292	114.6		
	3	0.2000	0.2270	113.5		
	4	0.2000	0.2295	114.7		
	5	0.2000	0.2265	113.2		
	6	0.2000	0.2041	102.0		
50%	1	2.4934	2.4928	100.0	100.0	0.7
	2	2.4934	2.5127	100.8		
	3	2.4934	2.4766	99.3		
100%	1	4.9868	4.9825	99.9	100.1	0.2
	2	4.9868	5.0002	100.3		
	3	4.9868	4.9992	100.2		
150%	1	7.4802	7.4982	100.2	100.2	0.3
	2	7.4802	7.4696	99.9		
	3	7.4802	7.5096	100.4		
200%	1	9.9736	9.9235	99.5	99.8	0.2
	2	9.9736	9.9400	99.7		
	3	9.9736	9.9690	100.0		
	4	9.9736	9.9581	99.8		
	5	9.9736	9.9536	99.8		
	6	9.9736	9.9740	100.0		
			Averag	103.4		
			%RSD	5.9		

Table 5: Precision results for Testosterone undecanoate.

No of injection	Area	Retention time
	Testosterone Undecanoate	Testosterone Undecanoate
Standard injection 1	2420828	27.600
Standard injection 2	2414432	27.627
Standard injection 3	2418284	27.653
Standard injection 4	2422922	27.660
Standard injection 5	2427131	27.680
Standard injection 6	2415976	27.680
Average	2419929	27.65
% RSD	0.2	0.1

Table 6: Ruggedness results of Testosterone undecanoate.

SampleName	Testosterone (%w/w)	Testosterone Decanoate (%w/w)	Highest Unknown impurity (% w/w)	Total Impurities(% w/w)
Sample1	1.0268	0.9423	0.1034	2.1932
Sample 2	1.0252	0.9412	0.1022	2.1906
Sample 3	1.0290	0.9490	0.1023	2.2028
Sample 4	1.0398	0.9617	0.1044	2.1872
Sample 5	1.0233	0.9475	0.1031	2.1966
Sample 6	1.0218	0.9416	0.1038	2.1884
Average	1.0277	0.9472	0.1032	2.1931
STD. Dev	0.0065	0.0078	0.0009	0.00582
% RSD	0.6	0.8	0.8	0.3

Table 7: Robustness results for Testosterone undecanoate.

Acceptance criteria	**The percentage relative standard deviation for peak areas of Testosterone Undecanoate obtained from six replicate injections of standard solution should be not more than 5.0**
Test Condition	0.6
Low flow variation (1.3mL/Minute)	0.2
High flow variation (1.7mL/Minute)	0.3
High column temperature Variation	0.4
Low wavelength variation (238nm)	0.3
High wavelength variation (242nm)	0.3
Low gradient (3%)	0.3
High gradient (3%)	0.9
Acceptance criteria	**The Tailing factor of Testosterone Undecanoate peak obtained from standard solution should benot more than 2.0**
Test Condition	1.0
Low flow variation (1.3mL/Minute)	1.0
High flow variation (1.7mL/Minute)	1.0
High column temperature Variation	1.0
Low wavelength variation (238nm)	1.0
High wavelength variation (242nm)	1.0
Low gradient (3%)	0.9
High gradient (3%)	1.0
Acceptance criteria	**The Theoretical plates of Testosterone Undecanoate peak obtained from standardsolution should be not less than 2000.**
Test Condition	60111
Low flow variation (1.3mL/Minute)	58116
High flow variation (1.7mL/Minute)	64228
High column temperature Variation	59151
Low wavelength variation (238nm)	61276
High wavelength variation (242nm)	61133
Low gradient (3%)	56748
High gradient (3%)	45684
Acceptance criteria	**The Resolution between Testosterone Decanoate and**

	Testosterone Undecanoate peaks obtained from the resolution solution should be not less than 2.0.
Test Condition	5.82
Low flow variation (1.3mL/Minute)	5.94
High flow variation (1.7mL/Minute)	5.50
High column temperature Variation	5.47
Low wavelength variation (238nm)	5.77
High wavelength variation (242nm)	5.77
Low gradient (3%)	5.67
High gradient (3%)	6.42

Table 8: LOD, LOQ of Testosterone undecanoate.

S. No.	**% Level**	**Concentration inμg/mL**	**Peak Response ofTestosterone**
1	0.02	0.220	141318
2	0.04	0.440	288335
3	0.10	1.100	724364
4	0.15	1.650	1092599
5	0.20	2.199	1442158
Slope			658773.5418
Intercept			-1213
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			0.9999
Standard Deviation			5428
LOQ (ppm)			0.082
LOD (ppm)			0.027

Table 9: Forced degradation study of Testosterone undecanoate.

For Stress Condition	**Peak Purity**			**% Degradation**
	A	**B**	**C**	
Unstressed Sample	1.0	1.0	1.0	0.2508
Added 2mL of 0.1N Sodium Hydroxidesolution kept in the water bath at 80°C for 30minutes and neutralized with 2mL of 0.1N Hydrochloric acid solution.	1.0	1.0	1.0	0.0859
Added 2mL of 3% hydrogen peroxidesolution kept in the water bath at 80°Cfor 30minutes.	1.0	1.0	1.0	0.0931
Added 2mL of water, kept in the water bath at 80°C for 30minutes.	1.0	1.0	1.0	0.0878
Photo stressed sample (open condition)	1.0	1.0	1.0	0.0919
Photo stressed sample (closed condition)	1.0	1.0	1.0	0.0941
Heat the sample kept in a hot air oven at 50ºC for 5 hours.	1.0	1.0	1.0	0.0757
Heat the sample kept in a hot air oven at 50ºC for 24 hours (with blister).	1.0	1.0	1.0	0.5894
Heat the sample kept in a hot air oven at 50ºC for 48 hours (with blister).	1.0	1.0	1.0	0.1236
Added 5mL of 4N Hydrochloric acid solution in the water bath at 80°C for 30minutes and neutralized with 5mL of 4N Sodium Hydroxide solution.	1.0	1.0	1.0	9.3671

A- Testosterone; B- Testosterone Decanoate; C- Testosterone Undecanoate

CONCLUSION

The developed stability-indicating Related Substances method for the determination of Testosterone Undecanoate and its degradation impurities using the RP-HPLC gradient method was found to be simple, accurate, precise, robust, rugged, and specific. Hence this method can be used for routine quality control and stability analysis. Identification and characterization of the impurities present may be taken up as further research in the study.

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