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Simultaneous determination of impurities and degradation products by rapid RP-UPLC-MS method

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ABSTRACT

The present article describes the development and validation studies on products of testosterone (Ts) in bulk drugs. The validation parameters include system suitability, specificity, accuracy, precision, linearity, limit of detection and limit of quantification. In addition to this, the stability of main drug has also been validated. Experiments have been carried out by using Ultra high Performance Liquid Chromatography, which is highly sophisticated and of superior technology instrumentation coupled to mass detector (UPLC-MS).Unlike HPLC, the products of forced degradation and impurities in testosterone can be accurately measured by using the mass detector in UPLC. Further Rapid analysis and high sensitive method was achieved by using hybrid technology column with linear gradient elution of mobile phase. The chromatogram was analyzed at 245 nm with a run time of 6.0 minutes. Linearity graph range was 10-150 % and trueness was at three levels of 50-150% of the specification limit. The detection and quantification limit of the method for impurities was 1.6 & 5 % of the specification limit. Test drug testosterone was found degradation towards the acidic, basic, thermal and oxidative conditions and was stable for other conditions as well, confirmed by mass detector. Application of the derived method over the older and slower HPLC methods was useful for a 10 fold increase in speed analysis of the test samples in various fields like process development R & D and quality control of bulk drug manufacturing. The study was analyzed as per ICH Q1A (R2) and Q2 (R1) guidelines.

Keywords: Testosterone, RP-UPLC, Impurities, Mass spectrometry, Degradation Products.

INTRODUCTION

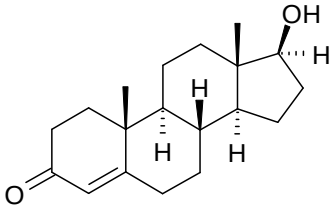
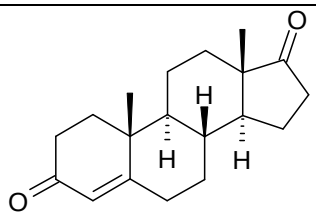
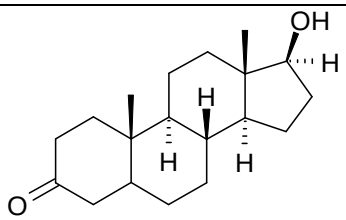
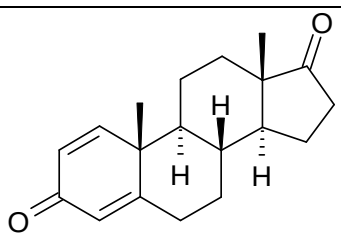
Testosterone (primary natural androgenic hormone), also called as androgens/steroids/anabolic steroids, is a male sex hormone that is essential for sexual and reproductive development. It is a primary testosterone that can be administered orally, transdermally or by injection for improving the hypogonadal sense of human beings, sexual functions, bone density and muscle strength as well [1]. Deficiency of testosterone affects approximately 30% of men aging from 40-79 years with an increase in prevalence, among people strongly associated with common medical conditions such as obesity, diabetes and hypertension [2].Testosterone hormones prevent abdominal obesity in middle aged men, gives beneficial effects on well being and is also involved in the cardiovascular and diabetes risk profiles. Similar sensations (results) were observed in postmenopausal women after hormone replacement therapy [3]. Testosterone measurement in the athletes of sports field and for animals in racing has nowadays attained greater attention.

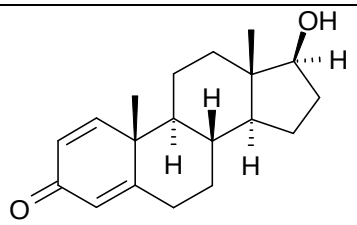
This article deals with the testosterone stability via impurity analysis by the application of Ultra Performance Liquid Chromatography (UPLC) coupled with single quadrupole mass detector. In Pharma industry, special emphasizes was given for analyzing the impurity profile of a drug by using different analytical techniques like Mass and Tandem Mass spectrometry, Fourier Transform Ion Cyclotron Resonance Mass spectroscopy (FT-ICR-MS), Nuclear Magnetic Resonance (NMR), Liquid and Gas Chromatography etc [4]. The various regulatory authorities such as (USP, BP, JP, IP and EP) are not only concerned with the purity profile but also on impurity profiling (identification, isolation and characterization of impurity) for obtaining approval of the license. International Conference on Harmonization (ICH) generally recommends the presence of impurities to be 0.1% and any level above it should be identified and quantified through appropriate analytical techniques.

As per the European monograph, Testosterone (Ts) has the Specification limit for each impurity should be not more than 0.6%. In that Impurity-F which has low UV was not detected by DAD and could be separated from other peaks, further confirmed by mass detector which shows the TIC (Total Ion Chromatogram) and mass spectrum m/z (291) with a retention time (RT) of 3.78 minutes. Very few analytical methods have been reported for the determination of Ts in bulk drug as well as in formulations viz., Impurity analysis of Testosterone by HPLC with 75 minutes run time [5], Light induced stability of Ts in different waters by LCMS [6], an LCMS method of Testosterone from thermo fisher scientific, assay of methyl testosterone by GC [7,8], near IR spectroscopy for the determination of testosterone [9], LCMS & GCMS methods for Ts determination in serum, urine, hair and plasma [10-13] and Spectrofluorimetric method for the Ts determination in biological fluids [14] etc. Also a review on forced degradation study, RP-HPLC Stability indicating assay method for Boldenone undecyclenate (Imp-H) and its related substances [15, 16] has been reported.

From the subsequent literature search, Testosterone was mainly determined by HPLC, GCMS and LCMS methods. It is well evident that an authenticated method is required for simultaneous determination of Ts in the presence of its related impurities with stability indicating parameters. Thus the present study deals with the same as per the ICH regulations [17, 18]. Using this rapid method, more number of routine samples could be analyzed with more quality and precision, it will be well suited in drug discovery (CRO) analytical department for routine synthetic R & D sample analysis with increased confidence by the appropriate incorporation of mass spectral data.

Table 1. Structural information of testosterone and its related impurities

S. No.	Name of the compound	Structure	Description
1	Testosterone		IUPAC Name: 17β-Hydroxyandrost-4-en-3-one Molecular Formula: $C_{19}H_{28}O_2$ Molecular Weight: 288.4 g/mol HPLC Purity: 99 %
2	Impurity-A		IUPAC Name: 4-Androstene-3,17-dione Molecular Formula: $C_{19}H_{26}O_2$ Molecular Weight: 286.4 g/mol GC Purity: 99.5 %
3	Impurity-F		IUPAC Name: 17β-hydroxy-5α-androstan-3-one Molecular Formula: $C_{19}H_{30}O_2$ Molecular Weight: 290.44 g/mol TLC Purity: 99 %
4	Impurity-G		IUPAC Name: 1,4-Androstadiene-3,17-dione Molecular Formula: $C_{19}H_{24}O_2$ Molecular Weight: 284.4 g/mol GC Purity: 99.3 %

5	Impurity-H		IUPAC Name: 17β-hydroxyandrosta-1,4-dien-3-one Molecular Formula: C19H26O2 Molecular Weight: 286.4 g/mol HPLC Purity: 99 %

MATERIALS AND METHODS

2.1 Chemicals and reagents:

Testosterone (17β-hydroxy-3-oxo-4-androstene) CAS no: 58-22-0, purity ≥99 % by HPLC and its known impurities 17β-hydroxy-5α-androstan-3-one (Impurity-F) CAS no: 521-18-6, purity 99% by TLC,17β-hydroxyandrosta-1,4-dien-3-one (Impurity-H) CAS no: 846-48-0 with purity 99 % by HPLC were purchased from Sigma Aldrich chemicals (Bangalore, India). Delta4-Androstene-3, 17-dione (Impurity-A) CAS no: 63-05-8, purity 99.5 % by GC and 1,4-Androstadiene-3,17-dione (Impurity-G) CAS no: 897-06-3, purity 99.3 % by GC are from TCI (Tokyo Chemical Industry)co., Ltd (Tokyo, Japan). Analytical grade chemicals and solvents, analytical-mass grade formic acid (Fluka) were purchased from Sigma-Aldrich Ltd. (Bangalore, India). Highly purified Milli-Q water used for the UPLC/MS from Milli-Q water purification system was procured from Millipore (Bangalore, India).

2.2 Standard & sample solution preparations:

Standard solutions of testosterone and its impurities were prepared by dissolving each 10mg of accurately weighed sample in 10mL volumetric flask using acetonitrile (100%). Test samples for validation were prepared from intermediate stock solution (50 ppm) obtained from main stocks using acetonitrile: water (9:1). The subsequent samples of linearity were prepared from the intermediate stock solution so as to obtain final concentrations of 10%, 25%, 50%, 100%, and 150%, with 1.65% and 5% of the specification limit (0.5% or 5 ppm is 100%) for LOD and LOQ with respect to API testosterone concentration (1000 ppm).

2.3 Instrumentation:

The chromatographic experiment was carried out using an Agilent UPLC system (Model No-1290 Infinity) consisting a binary pump (capable of producing pressure up to 15000 psi) with high pressure gradient having low delay volumes. UPLC, an innovative separation technique decreases the analysis duration drastically over the HPLC, connected to a highly sensitive DAD detector (very narrow flow cell path length). The use of hybrid technology column with sub 2 micron particle size ($C_{18,}$ 50x2.1mm, 1.7µm) offers significant advantages over present HPLC instruments. The current research was coupled with Mass detector SQD-6190 (Single Quadrupole Detector) having ESI source, wherein the results are obtained using the software Chemstation version-C.01.04 (35). By using mass spectrums (m/z) of testosterone, impurities and degradation products arising from forced degradation at both modes (ESI +ve and –ve modes) was obtained that was helpful in identification of the nature of molecule at various conditions.

Mettler Toledo made in Switzerland analytical balance and Bandelin Ultrasonicator were also used for this study.

2.4 Chromatographic method:

The analysis was completed with C18 material Acquity BEH column (50x2.1mm, 1.7µm) using the mobile phase [0.05% formic acid in water as aqueous buffer (%A) and 0.05% formic acid in acetonitrile as organic solvent(%B)] sonicated for 10 minutes and filtered through 0.45µm membrane filter. To get good separation of testosterone from its known impurities and degradative products, a linear gradient program (%B: 0/25, 5/50, 5.5/25, 6/25, 6.01/25) at a flow rate of 0.5mL/min was used. Before running the instrument, the column was equilibrated for 10 minutes with an initial gradient flow. Aliquot samples of 2µL were then injected into the column, kept at 35 ^{0}C column oven temperature.

Mass spectrometry Detection parameters were: Ion source (ESI) spray voltage (4 k volts for +ve and –ve polarity), drying gas temperature of 300^0 C. Furthermore, nebulizer pressure (55 psig) and drying gas (10L/min) were used in mass spectrometry. The ions from mass detector were monitored in full scan mode in the mass range of 100-1000 m/z.

RESULTS AND DISCUSSION

Today pharmaceutical industry faced major challenges are finding different techniques and new ways to enhance productivity, reduce costs whilst still ultimately developing new therapies that improve human health. Earlier

investigations that were performed with chromatographic conditions were costlier and time consuming with low productivity. They also have higher flow rates, more run time and pH adjustment of mobile phase. In comparison, the developed test method adopted for analyzing the impurity profiling of testosterone was simple, rapid and cost effective, so that more no of method optimization trials should possible with lesser run times.

Initial studies were tried with water, acidic, neutral and basic buffers using different dimension of columns. It was found that in neutral buffer (5mM Ammonium Acetate: Acetonitrile) with C18, an irregular peak with poor resolution & low ionization in mass spectrometry were observed, whereas in the basic buffer (5Mm Ammonium bicarbonate: Acetonitrile) with C18, a good peak shape was observed along with low sensitivity. In case of water as mobile phase, it gave symmetrical peak shape and resolution but lacked reproducibility. Finally, high quality chromatographic & spectrometric results were observed using BEH-C18 column (50x2.1mm, 1.7 µm) with 0.05% formic acid in water and acetonitrile. Using this developed method, the drug testosterone and its impurities were well separated from its degradative products generated from forced degradation. The results of this test method are discussed below.

3.1 System Suitability:

System suitability was performed by running five injections of standard solution of Ts spiked with four known impurities repeatedly and the system was verified. Freshly prepared working solutions from stock solution were used for UPLC injections. The system performance was checked by acquiring the results and characteristic parameters such as resolution, tailing factor, theoretical plates, retention time and relative standard deviation which were found to be within their permissible limits. (Table: 2). The exact amount of impurities were obtained from the relative response factor calculated by multiplying with correction factor of known individual impurities, in this case it was found that drug Ts and three impurities, Imp-A, Imp-G, Imp-H were possessing the same UV maximum at 245nm.

Table: 2 Comparative results of System Suitability (n=5)

Parameters	Testosterone	Imp-G	Imp-A	Imp-H
% RSD Standard area	0.21	0.65	0.63	0.59
% RSD RT	0.20	0.00	0.14	0.20
Theoretical plates	21455	16910	32912	8950
USP tailing	1.05	1.07	0.99	1.06
Resolution	2.69	6.05	4.01	-
Selectivity	1.08	1.16	1.16	-

3.2 Specificity:

Specificity of the chromatographic method was established by separation of drug peak from the adjacent resolving peaks or from its potential impurities. Specificity was verified by two parts, Specificity-part-A and part-B.

Part-A was run using a blank, drug (Ts) and its known impurities working solution, to check separation and resolution. In part-B, specificity was confirmed by inducing degradation of testosterone to acidic, basic, neutral, peroxide, UV chamber and temperature. For all degradation, the analyte concentration was maintained at 1 mg/mL and for thermal and photolytic-UV chamber, dry state sample was taken. The peak purity and mass purity of principle peak was established using DAD & mass detector which has shown efficient separation of drug (Ts) peak from its degradation peaks.

3.3 Accuracy:

Accuracy expresses the closeness of individual measured value to the nominal value. The % recovery of the developed test method was analyzed to known impurities by spiking these related substances solution prepared from blend stock solution to drug(Ts) sample solution (1000 ppm), performed at 50%, 100% and 150% levels with respect to specification limit for each impurity in triplicates (Table 3). The recovery calculations are done by area normalization method and compared with standard impurities solutions of that particular level (50%, 100% and 150%). Results of the recovery study for related substances were found to be within the permissible limits of 92.16% to 103.61%.

Table: 3 Results of Accuracy-Recovery Study

IMPS	IMP-G			IMP-A			IMP-H		
	50%	100%	150%	50%	100%	150%	50%	100%	150%
Average Area of IMPS(*)	83.88	171.32	252.01	51.21	104.54	154.95	35.09	71.90	106.13
Average Area of IMPS with API(*)	77.31	163.65	245.39	47.14	100.45	160.22	33.40	69.21	109.97
Mean % Recovery	92.16	95.53	97.37	92.05	96.09	103.41	95.18	96.26	103.61

***Each value is a mean of three readings, IMP: Impurity*

3.4 Precision:

Precision expresses the repeatability of same homogeneous sample solution. The present study was articulated by injecting five individual preparations of Ts (1000 ppm) solution spiked with known impurities (Imp-A, Imp-F, Imp-G and Imp-H) at 100% specification limit. The Intermediate precision or ruggedness was evaluated on different days, with different instrument, analyst and reproducibility was done on the same day. The results of the precision study are shown in table 4. The % RSD of the total precision study was found to be 0.228-1.656.

Table: 4 Results of Intra-day and intermediate precision of Ts and its RS (%RSD of n=5 injections of Test concentration)

Compounds	Intra-day precession		Intermediate Precession
	System Precession(n=5)	**Method Precession**(n=5)	**Different day/Different Instrument**(n=5)
Ts	1.163	0.226	1.12
IMP-G	0.228	1.656	1.47
IMP-A	0.425	1.469	0.25
IMP-H	0.775	1.416	0.73

3.5 Limit of detection (LOD) and limit of quantification (LOQ):

LOD and LOQ were evaluated for method sensitivity performed by injecting diluted known concentration of the solution that gives minimum detectable peak area. This was multiplied thrice to get LOD, within the detectable limit and quantified as an exact value 10 times to get LOQ with suitable precession as per International Conference on Harmonization guidelines Q2 (R1)(18). From the results obtained, the projected method quantified small quantity of impurities in Ts samples. LOD and LOQ values for related substances (Imp-A, Imp-G, and Imp-H) were found to be 0.08 and 0.25 ppm respectively, the % RSD of LOQ was 3.03-9.53%.

LOD and LOQ calculations were done with the following equations.
$LOD=3\sigma/s$ and $LOQ=10\sigma/s$
(σ is the Standard deviation of intercept; s is slope of linearity curve)

3.6 Linearity:

The Linearity of impurities (Imp-A, Imp-G and Imp-H) was evaluated by related substance method in the range of LOQ (5%) to 150% of the specification limit. The samples were prepared from blend stock solution (50 ppm) using diluents, injected into the UPLC-MS system keeping the injection volume of 2µL constant. The obtained correlation coefficient was greater than 0.995 to 0.997. Linearity was monitored for related impurities for two days. From the results, it was concluded that there existed excellent correlation between sample concentration and peak area for all known impurities. The values of correlation coefficient slope and Y-intercept for all related substances are tabulated in table 5.

Table: 5 Results of Linearity (n=3)

Parameters	Imp-G	Imp-A	Imp-H
Lin-Range(µg/mL)	0.25-7.5	0.25-7.5	0.25-7.5
R2	0.996	0.995	0.996
Slope	1.923	1.178	0.807
Intercept	-0.313	-0.112	-0.526

3.7 Robustness:

The robustness of chromatographic method was evaluated by changing deliberate experimental conditions like flow rate, buffer concentration (strength), wavelength and column temperature and the resolution were recorded for API (Ts) and its known impurities. The present study was performed by changing the flow rate ($\pm$0.02 mL/min), mobile phase concentration of formic acid ($\pm$0.01%) and column oven temperature ($\pm$2 ^{0}C). A small difference in peak areas, retention times were observed for flow rate changes, insignificant difference for mobile phase concentration and column temperature. Resolution was calculated for the main peak and the closest impurity i.e. impurity-A obtained by running the system suitability concentration.

Table: 6 Results of Robustness

Parameters	Flow rate (mL/min)			Buffer conc. (%)			Column temp (^{0} c)		
	0.48	0.5	0.52	0.04	0.05	0.06	33	35	37
Resolution between API and Imp-A	6.12	6.06	6.10	6.10	6.06	6.15	6.26	6.06	6.03

3.8 Solution Stability:

It is required to demonstrate stability of the sample in solution for generating reliable results for validation. Stock solution stability was checked at 2-8 ^{0}C refrigerated for 8 days, bench top at room temperature for 8 hrs and for auto sampler is 12 hrs, compared with freshly prepared sample solution.

Table: 7 Results of Stock solution stability (n=5)

Sr. no	Parameter	%Purity	%Bias
1	Fresh Sample	99.91	-
2	2-8^{0}c@8 Days	99.89	-0.02
3	Auto Sampler@12 hrs	99.99	-0.01
4	Benchtop@8 hrs at RT	99.86	-0.05

3.9 Forced Degradation studies:

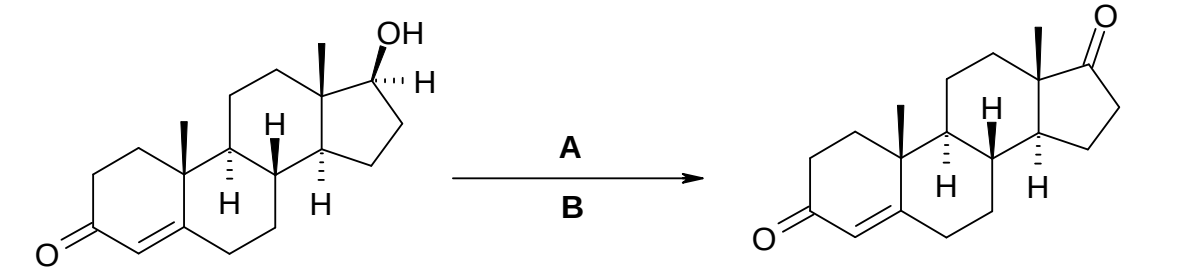
It is a procedure, where the usual degradation rate of drug substance is accelerated by the application of additional stress. In order to attain it, induced degradation has been carried out to confirm that during stability study or all throughout the shelf life, any induced product from degradation if found, will not interfere in the main peak and with known impurities. In addition, forced degradation study will help to identify the type of degradation pathways (such as acidic, alkali, oxidative, photolytic and dry heat) for each of the degradants [19].

The guidelines of ICH demonstrated that induced degradation was planned to determine the stability of a drug product by expressive degradation pathways. In order to recognize the degradative products, this degradation study covers a wide range of pH. Nowadays, regulatory authorities (ICH & FDA) are rising to include this study at Phase-III stage, to get valuable information about the drug substance for its formulation development and manufacturing process [20, 21].

The present study was run with mass spectrometer which was more helpful in confirming the pathway of degradation behavior of drug at different environments obtained by identifying the degradative mass spectrums and elucidating the possible structure of degradation products. The test drug (Ts) was easily converted to Dione of Imp-A (Adrostenedione) at oxidative and thermal degradation, it was confirmed by mass to charge ratio and retention time of Imp-A, as described in the reaction scheme below. Major unknown impurities were found in base and acid degradation, at acidic media drug Ts was decomposed to 5.70% with m/z ratios of 246(-43), 330(+41) and 288(-H), illustrating that the drug (Ts) might have lost hydrogen thereby giving peak at 288 m/z. In basic degradation, the induced products m/z ratios were 349, 318(+29) and 335(+46). The degradation details were listed in table 8.

Table: 8 Results of Forced degradation

Stress conditions	% Degradation	+ve mode(m/z)	-ve mode(m/z)
Treated with 6N HCl solution for 6 hrs (sonication) on a water bath at 80 °C.	5.70	246,330,288(-H)	-
Treated with 5N NaOH solution for 6 hrs (sonication) on a water bath at 80 °C.	24.09	349,318,335	289,333
Treated with milli-Q-water 6 hrs (sonication) on a water bath at 80 °C.	1.57	282	-
Treated with 10% H_2O_2 solution 6 hrs (sonication) on a water bath at 80 °C.	11.50	287(Imp-A)	-
Treated with heat at oven about to melting point for 3hrs.	39.94	303,301,287(Imp-A)	-
Exposed to UV light for 24 hrs (≥200 w h/m^2).	0.27	-	-



Testosterone (Mwt: 288.4) **Impurity-A (Mwt: 286.4)**

A). 10% H2O2 solution 6 hr on a water bath at 80 °C.
B). Heat in oven about 100 °C for 4 h.

Respective Chromatograms:

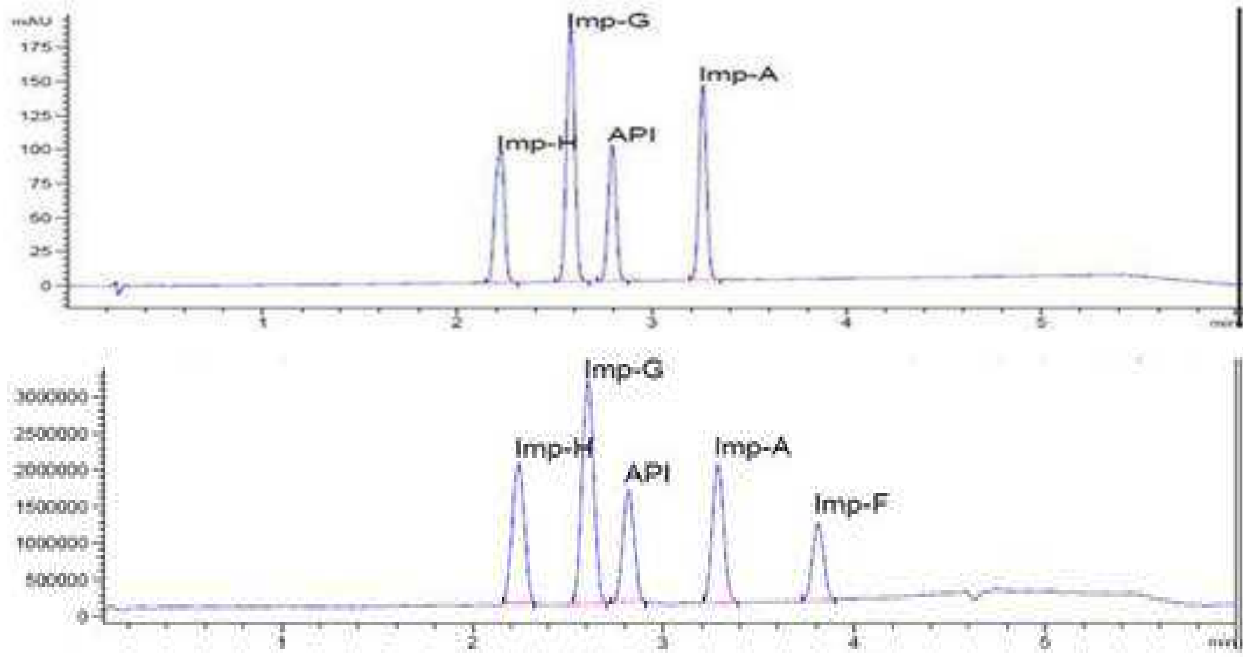


Fig: 1 Chromatogram & TIC of Testosterone and its impurities.

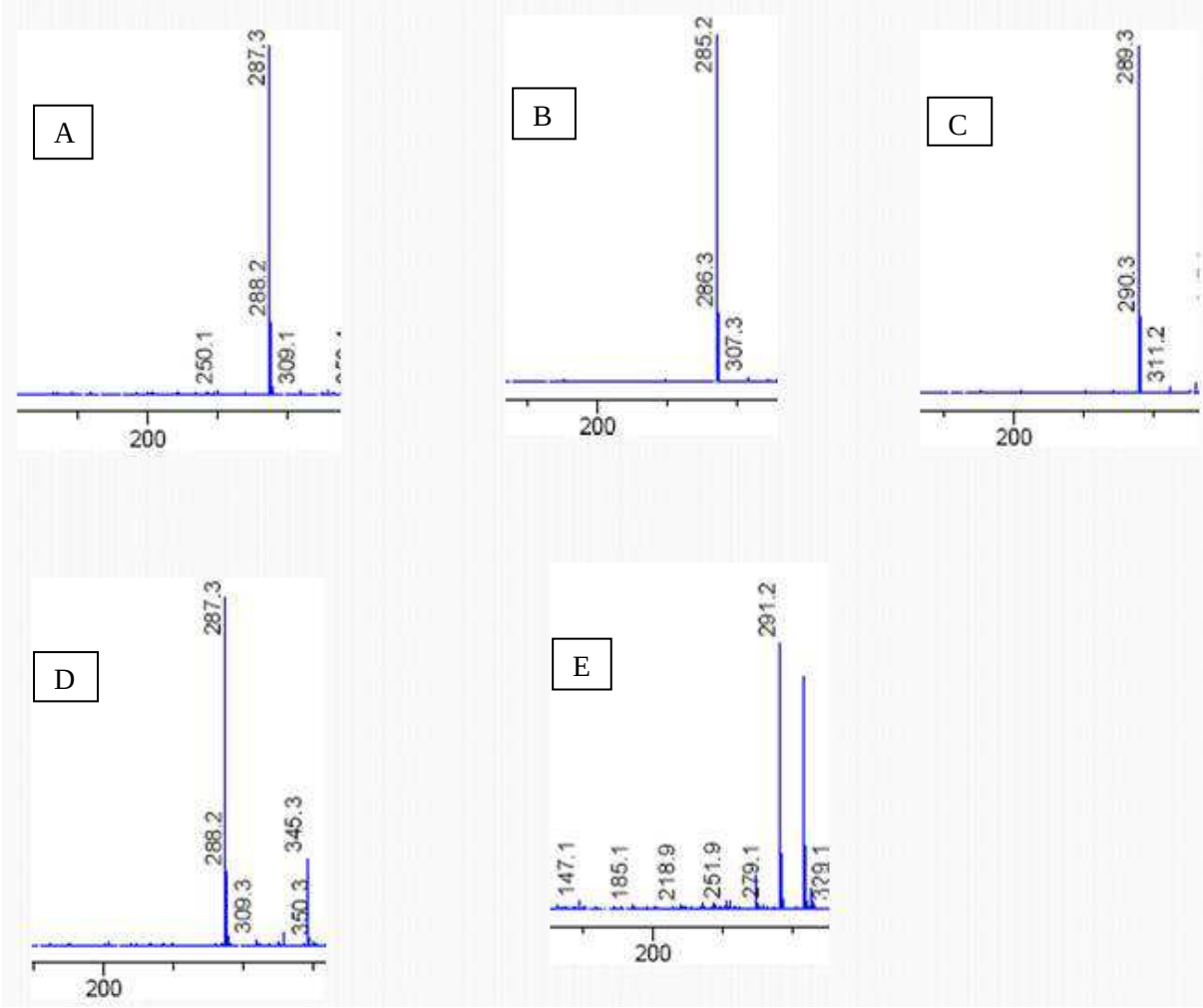


Fig: 2 Mass spectrums of A, B, C, D, E is Imp-H, Imp-G, API(Testosterone), Imp-A & Imp-F

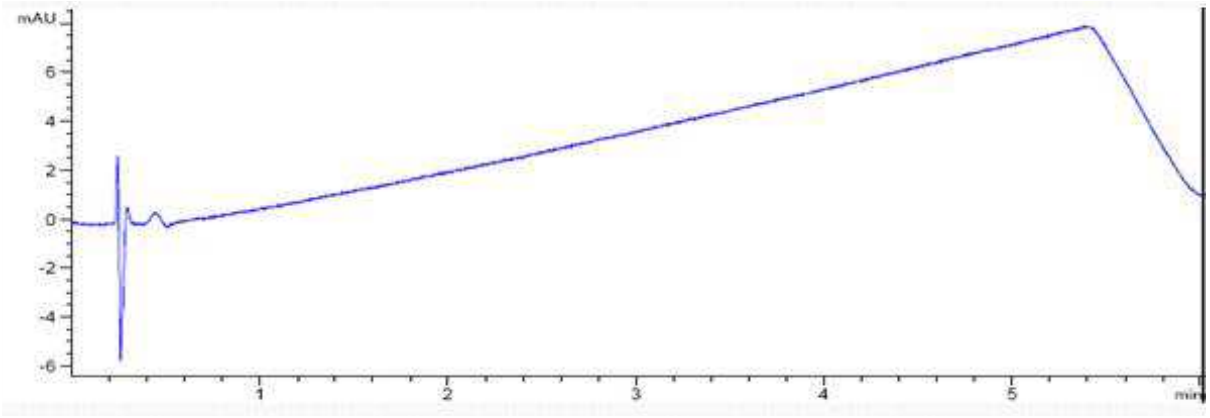


Fig: 3 Chromatogram of blank (diluent)

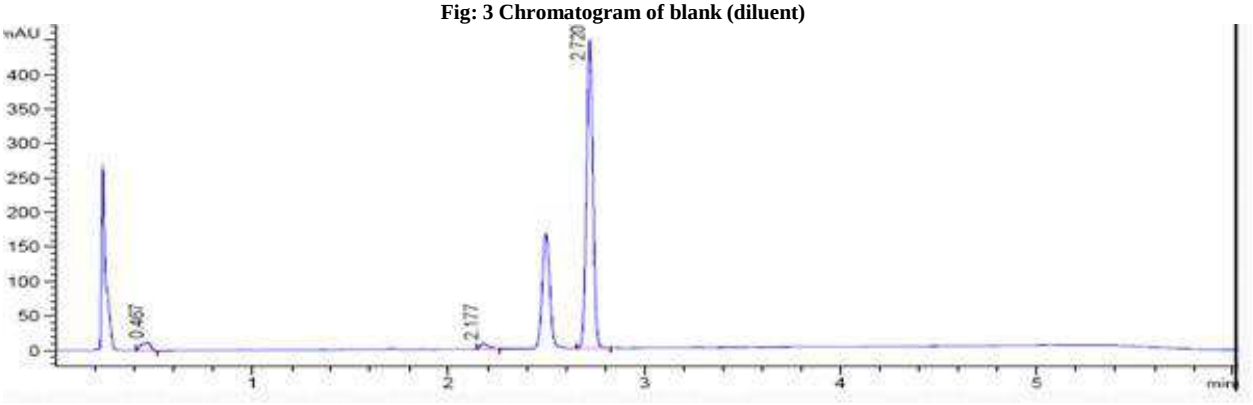


Fig: 4 Chromatogram of Acid degradation with 6N HCl in water bath 6hrs at 80^0 C

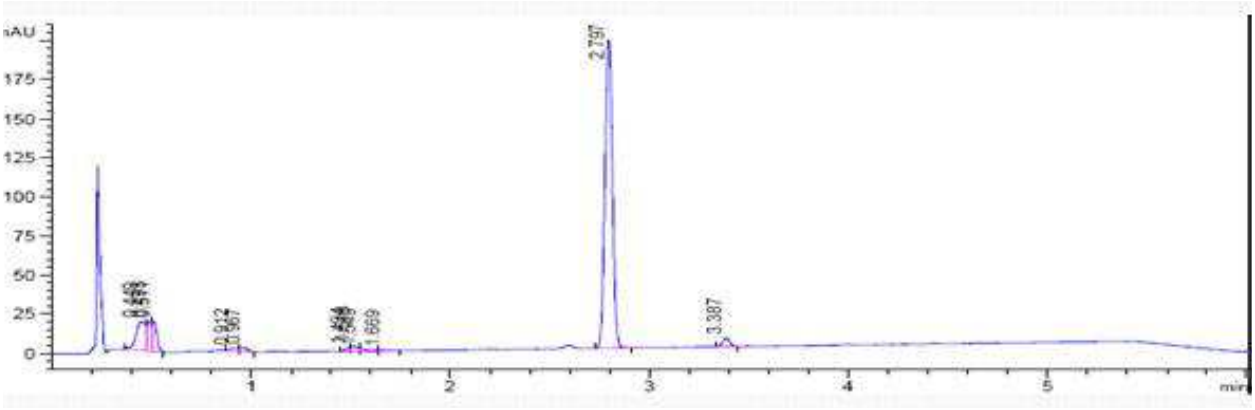


Fig: 5 Chromatogram of Base degradation with 6N NaOH in water bath 6hrs at 80^0 C

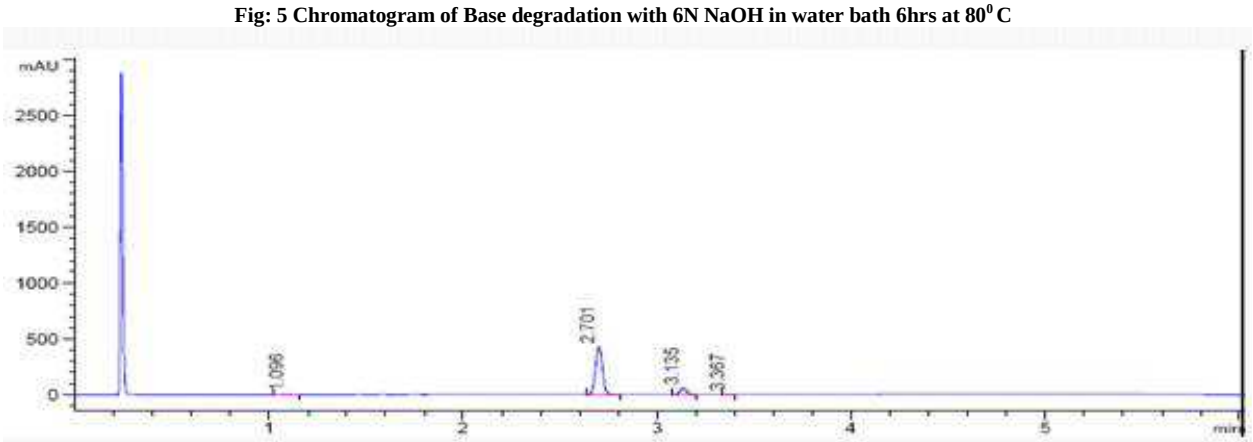


Fig: 6 Chromatogram of Oxidative degradation with 10% H2O2 in water bath 6hrs at 80^0 C

CONCLUSION

In this study, the present method elaborates the need for estimation of process related substances in testosterone by using UPLC-MS, which is simple, sensitive, accurate, precise and reliable ICH validated method with minimal run time. By this method, the test drug, its process related impurities and all the degradative products from forced degradation were well separated, which were well identified by mass spectrometry. Hence, UPLC-MS method could find an application as a convenient technique for the ongoing process control analysis in bulk drugs and for impurity profiling in analytical research department (API-Pharma Industry) as a time saving (only 6 minutes are needed for the analysis one sample) and more productive. Additionally, the test method can easily be extended for the analysis of a variety of steroid hormones because of the sensitive mass detection method. It is concluded that the current method is eco-friendly for humans as well as for the environment. Further the developed test procedure also used to perform assay of drug product.

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