Original Article

HPLC Method Development and Validation for the Quantification of Related Impurities in Testosterone Cypionate Active Pharmaceutical Ingredient

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ABSTRACT

Aim: The purpose of this research study, is to develop and validate a reverse phase HPLC test method for detecting relevant impurities in Testosterone cypionate (TCY). **Materials and Methods:** The chromatographic system for separation of related impurities were achieved in Zorbax XDB-C8 (15 cm x 4.6 mm), 5 micron HPLC column utilising gradient elution technique. Water was selected as solvent-A and Acetonitrile was preferred as solvent-B for mobile phase. The method is gradient technique. Column heater was kept constant at 35°C; the rate of flow was 1.2 mL per min; volume of injection was 20 μL and 240 nm was set for detector wavelength. **Results:** The % recovery was in the range of 95.6% to 108.7% for all impurities. The result of correlation coefficient were higher than 0.98. Testosterone is the major degradants obtained from forced degradation study. **Conclusion:** The created method can be utilise in quality control testing on a regular basis for the analysis of Testosterone cypionate.

Key words: Testosterone cypionate, Testosterone replacement therapy, Male hypogonadism, HPLC, Impurity profiling.

INTRODUCTION

Testosterone is a natural sex hormone in male that helps males to develop their sex organs and improve their secondary sexual characteristics. TCY (testosterone cypionate) is an anabolic-androgenic steroid and manufactured by esterifying Testosterone. This type of product has a higher lipid solubility, which delays medication release into circulation and improves pharmacological action.[1] TCY is commercially known as Depo-testosterone and available as an oily based injectable solution.[2] Approximately 200 mg of TCY is injected intramuscularly ones in every 2 to 3 weeks to compensate the serum testosterone level, which occurs due to improper function of testis, results in male hypogonadism.[3-6] It is commonly used to treat male hypogonadism, which causes erectile dysfunction, delayed puberty, osteoporosis, a decline in ejaculate volume, and an rise in body fat.[7-12]

Several scientific papers are published on the applicability of several assement techniques for the detection and monitoring of anabolic-androgenic steroids and its metabolite in biological matrices.[13-17]

HPLC method development and validation for testosterone Cypionate, Propioante and other testosterone esters in drug substance and oil based injectables have also been studied.[18-20] In addition, the assay method for testosterone cypionate by Gas chromatography with Flame ionization detector has been described in United States Pharmacopoeia.[21] Various pharmacopoeias such as IP, Ph.Eur, USP and JP have yet not reported the chromatographic HPLC test method for the assessment of related impurities in

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TCY. Hence it is critical to establish stability reflecting, specific and accurate test method for the assessment of possible impurities in TCY. Current project describes the development of an HPLC technique for impurity profiling, forced degradation, and TCY method validation in accordance with current ICH criteria.[22-25]

MATERIALS AND METHODS

Materials and chemicals

Sample of TCY and Imp-1 to Imp-6 were obtained from Ipca laborotaries Ltd. Water for the analysis was acquired from Millipore's Milli-Q plus water purification equipment (HPLC grade) (Bedford, USA). Merck India supplied the acetonitrile (Mumbai, India).

HPLC conditions

Waters HPLC (2695) with UV detector (2487) and PDA detector (2996) (Waters Corporation, USA); a photo stability chamber model NEC-104RTS (Newtronic, Mumbai, India), where utilise for the study.

In this study, the chromatographic column Zorbax XDB-C8 (15 cm x 4.6 mm), 5 micron was utilised (Agilent, santa Clara, United States). Water was selected as solvent-A and Acetonitrile was preferred as solvent-B for mobile phase. The method is gradient technique. Column heater was kept constant at 35°C; the rate of flow was 1.2 mL per min; volume of injection was 20 µL and 240 nm was set for detector wavelength and the sample temperature was 25°C. The diluent was a 30:70 (v/v) mixture of water and acetonitrile. Table 1 shows the gradient programme.

Preparation of sample, stock solution for validation and forced degradation samples

Transferring around 20.0 mg of TCY sample into a 50 mL volumetric flask, 10 mL of acetonitrile was added, sonicated for 1.0 min, and made up to the volume with diluent yielded a 400 µg/mL test sample of TCY. Transferring around 20.0 mg of TCY sample and all impurities (imp-1 to imp-6) into 7 separate 25 mL volumetric flasks, adding 10 mL of acetonitrile, sonicating for 1.0 min, and making up to the volume with diluent yielded an 800 µg/mL individual stock solution. 0.75 mL of each of the above separate stock solutions was transferred to a 20 mL volumetric flask and diluent was added to make up the volume. Standard stock solution (30.0 µg/mL) was indicated on this solution. Other required solutions of various concentrations were created for validation using above standard stock solutions.

Each sample weighed around 20 mg and was divided into three 50 mL volumetric flasks designated 1, 2, and 3. Into each volumetric 10 mL acetonitrile was added and the sample was dissolved. In volumetric flasks 1, 2, and 3, 5 mL of 0.5 N hydrochloric acid solution, 1 mL of 0.5 N sodium hydroxide, and 2 mL of 5.0 percent hydrogen peroxide solution were added, respectively. The flasks were held at 60°C in a water heater bath for 2 hr, 20 min, and 4 hr, respectively. In volumetric flasks 1 and 2, the excess acid or base was neutralised and diluent was added up to the mark. Blank solutions were generated in the same way. Thermal deterioration of a solid sample was carried out at 80°C for 48 hr. Photolytic deterioration was achieved by spreading the sample on a petri dish and exposing it to 1.2 million Lux hours of white fluorecent light and 200 Wh/m2 of Ultra violet light in a photo stability chamber.

Table 1: Linear gradient program.

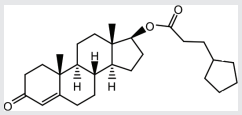
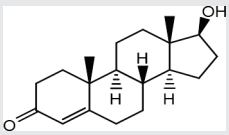
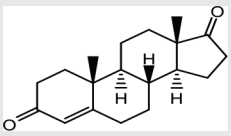
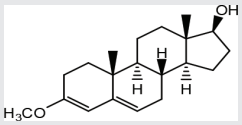
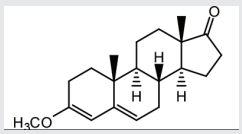
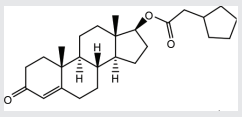
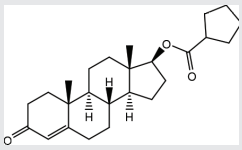
Time/minutes	Solvent-A, %	Solvent-B, %
0	50	50
5	50	50
20	20	80
30	20	80
35	50	50
45	50	50

RESULTS AND DISCUSSION

HPLC method development

The goal of the chromatographic procedure was to obtain a baseline separation between all relevant impurities and to elute TCY before 30.0 min in order to shorten the time duration. The elution time of impurities was used to designate them. Structural details of TCY and its impurities are listed in Table 2. TCY and its possible potential impurities have a maximum wavelength of 240 nm as shown in Figure 1. The method development was initiated using water and acetonitrile in a combination of (50:50, v/v) by isocratic elution method, using Zorbax XDB-C8 (15 cm x 4.6 mm) 5 micron column. The peak of TCY was not eluted upto 100 min. The mobile phase was then modified to water and acetonitrile in a combination of (40:60, v/v) in which the peak of TCY elutes at about 47.0 min. After multiple experiments, the analysis methodolgy was finally developed using linear gradient program. Water was selected as solvent-A and Acetonitrile was preferred as solvent-B for mobile phase. Both the mobile phase is utelise in gradient elution procedure. The column heater was kept constant at 35°C; the rate of flow was

Table 2: Structure and details of TCY and its impurities.

S.no	Name	Structure	Code	Source
1	Testosterone Cypionate		TCY	Drug
2	Testosterone		Imp-1	Process and degradation Impurity
3	Androstenedione		Imp-2	Process impurity
4	3-Methoxy testosterone		Imp-3	Process impurity
5	3-Methoxy Androstenedione		Imp-4	Process impurity
6	Testosterone Cyclopentane acetate		Imp-5	Process impurity
7	Testosterone Cyclopentane Carbonate		Imp-6	Process impurity

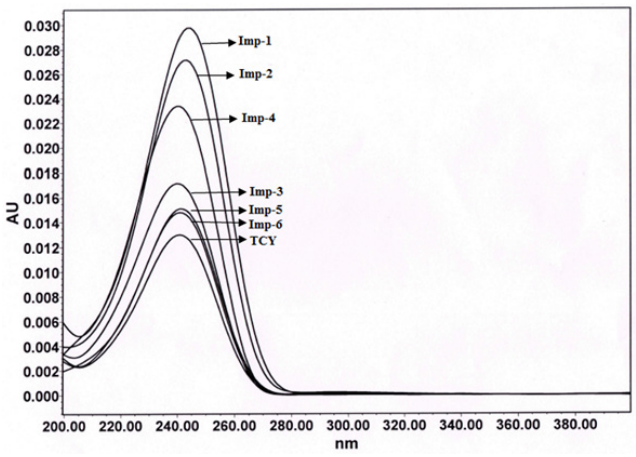


Figure 1: The λ_{max} of Testosterone Cypionate and its potential impurities.

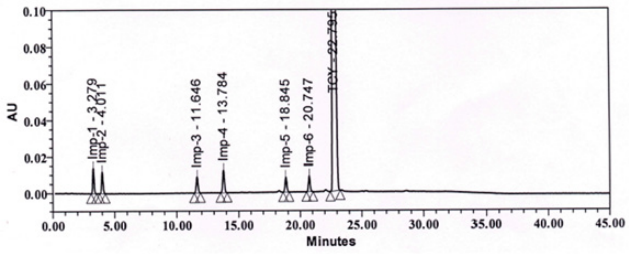


Figure 2: Chromatogram of spiked impurities in Testosterone Cypionate.

1.2 mL per min; volume of injection was 20 µL; 240 nm was set for detector wavelength and the sample temperature was 25°C. The TCY peak eluted at about 22 min with the base line separation of all impurities (Figure 2).

Forced degradation study

TCY was forced to detoriate in acidic, basic, thermal, oxidative, and photolytic conditions. TCY was degrade in both acidic and basic environments. Imp-1 was

identified as the impurity generated in both situations. TCY was unaffected by oxidative, thermal, or photolytic conditions. The peak purity results of the analyte peak obtained from the Photodiode array detector in all the stress samples confirmed that method is stability indicating. The chromatograms are showed in Figure 3 and data is tabulated in Table 3.

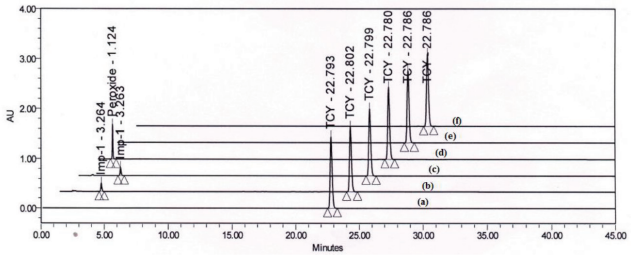


Figure 3: Chromatogram of forced degradation studies of Testosterone Cypionate: (a) control sample, (b) acid hydrolysis, (c) base hydrolysis, (d) oxidative degradation, (e) photolytic degradation, (f) thermal degradation.

Table 3: Percentage of degradation under different conditions.

Condition	Degradation %
Acidic (5 mL of 0.5N HCl, 60°C, 2 hr)	6.63
Basic (1 mL of 0.5N NaOH, 60°C, 20 min)	7.43
Oxidation (2 mL of 5% H2O2, 25°C, 4 hr)	No degradation
Thermal (80°C, 48 hr)	No degradation
Photolytic (Photolytic chamber, 10 days, 25°C)	No degradation

Method validation

The method must be verified according to ICH criteria to ensure specificity, solution stability, sensitivity, linearity, precision, accuracy, and robustness. Table 4 contains a summary of the method validation results.

Specificity

One of the most significant parameters in HPLC method validation is specificity. It refers to the analytical method ability to distinguish between the analyte and other impurities in the sample. The specificity of the procedure was tested by injecting a blank, sample solution, and sample added with potential impurities independently. All six impurities are well segrigated from each other and TCY peak. Also, no blank interference was observed. Stress degradation analysis was also performed to assess the proposed method specificity and stability indicating properties.

Solution stability

By injecting sample solution and sample added with related impurities in a tightly closed HPLC vial at 25°C in a temperature controlled automated sampler, the solution integrity of TCY and impurities were investigated. Content of each impurity was checked for initial (0 h) and after 24 hr and the percentage difference found was less than 10.0 %, demonstrating the stability of the solution upto 24 hr at 25°C in the recommended diluent.

Detection limit (LOD) and quantitation limit(LOQ)

The sensitivity of the procedure is described by LOD and LOQ limit. The lower the value, the more sensitive

Table 4: Method Validation summary data.

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	TCY
Retention Time (RT)	3.28	4.01	11.65	13.78	18.85	20.75	22.80
Relative RT	0.14	0.18	0.51	0.60	0.83	0.91	1.0
Resolution	--	3.37	30.71	8.10	19.52	7.41	7.49
Symmetry factor	1.17	1.14	1.06	1.07	1.10	1.09	1.20
Response factor	0.76	0.74	0.83	0.61	0.94	0.99	1.0
Linearity	0.9999	0.9997	0.9999	0.9998	0.9999	0.9998	0.9999
Detection Limit (µg/mL)	0.0358	0.0359	0.0357	0.0358	0.0354	0.0351	0.0359
Quantitation Limit (µg/mL)	0.1192	0.1198	0.1192	0.1194	0.1184	0.1171	0.1197
Intra-day precision (*n*=6, % RSD)	0.56	0.50	0.78	0.35	0.76	1.39	--
Inter-day precision (*n*=6, % RSD)	0.69	0.66	0.56	0.67	0.35	0.68	--
Accuracy at QL level	100.1	98.9	99.0	95.6	103.4	105.7	--
Accuracy at 100 % level	99.6	101.1	100.9	99.3	101.1	102.7	--
Accuracy at 150% level	105.4	104.5	104.5	102.5	102.4	108.7	--

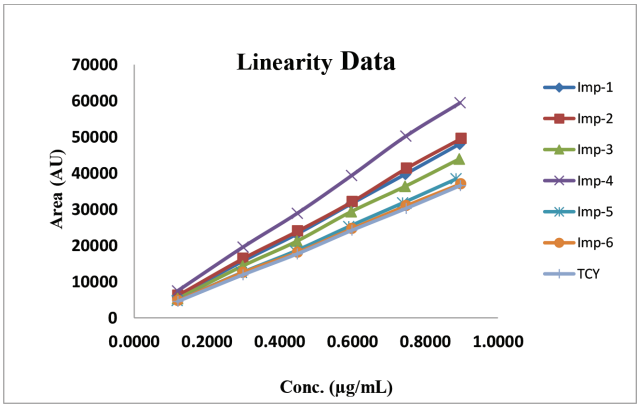


Figure 4: Linearity data of all impurities and Testosterone Cypionate.

Table 5: Batch results.

Name of impurity	Batch-1	Batch-2	Batch-3
Impurity-1	0.12	0.07	0.10
Impurity-2	0.05	Not detected	Not detected
Impurity-3	0.07	0.04	Not detected
Impurity-4	Not detected	0.05	0.03
Impurity-5	0.07	0.04	0.07
Impurity-6	0.09	0.08	Not detected

the procedure. The LOD and LOQ for each impurity as well as TCY were calculated and found in between 0.0351 µg/mL to 0.0359 µg/mL and 0.1171 µg/mL to 0.1198 µg/mL respectively.

Linearity and range

The lease square approach was used to determine linearity for all specified impurities and drug substances from the LOQ level to the percentage of specified limit of the analyte concentration (0.4 mg/mL). The standard stock solution was further diluted to the necessary concentrations and created six different concentration. The correlation coefficients determined for all impurities were greater than 0.98, indicating a positive link between peak areas and impurity concentrations (Figure 4).

Accuracy

Recovery tests were used to determine the accuracy of the related substances technique. Spiking known amounts of impurities in a TCY sample (400 µg/mL) in triplicate at the LOQ level (0.03 percent of the drug substances), at 100 percent (0.15 percent of the drug substances), and 150 percent (0.225 percent of the drug substances) was used to determine accuracy. The added concentration was compared to the recovered concentration to determine the recovery percentage for each impurity. The recovery percentage ranged from 95.6 percent to 108.7 percent.

Precision

Six independent preparations of TCY samples (400 µg/mL) spiked with 0.15 percent (0.60 µg/mL) of each known impurity were used to achieve the precision of the analytical test method (within the same day). The inter-day precision was established by performing analysis by another analysts on another instruments and on separate day. In both the precision, the percentage RSD for each impurity was found to be less than 10.0 percent.

Robustness

As per the study, even purposely adjusting the chromatographic settings (i.e. column heater, rate of flow and mobile phase composition) had no effect on the procedure. The resolution of all impurities and the TCY peak was found to be more than 2.0. This demonstrated the robustness of the method.

Batch results

Three distinct batches of testosterone Cypionate samples were subjected to the proposed HPLC technique. Imp-1 and Imp-5 was found in all the batches. The results are tabulated in Table 5.

CONCLUSION

The HPLC test method with UV detector for the assessing of possible impurities in Testosterone Cypionate (TCY) reported in this paper is simple, precise, sensitive, accurate and linear. The validation was carried out in compliance with ICH criteria and the test method can now be utilised for routine and additional stability analysis of testosterone cypionate drug substances.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

TCY: Testosterone Cypionate; **HPLC:** High Performane Liquid Chromatography; **USP:** United States Pharmacopoeia; **Ph.Eur:** European Pharmacopoeia; **JP:** Japanese Pharmacopoeia; **IP:** Indian Pharmacopoeia; **ICH:** International Council for Harmonisation of Technical Requirement for Pharmaceuticals for Human use; **UV:** Ultraviolet; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **RSD:** Relative Standard deviation.

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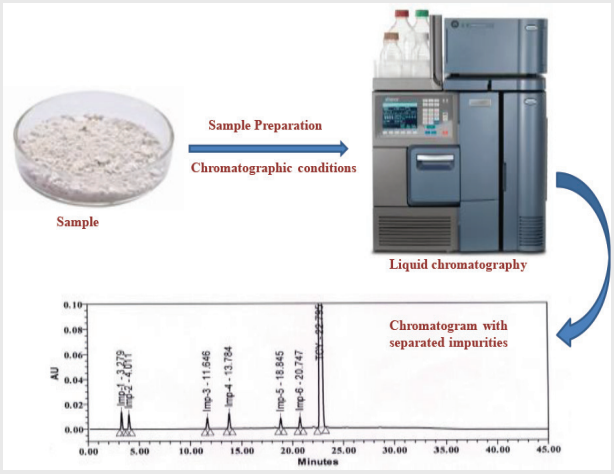
PICTORIAL ABSTRACT



SUMMARY

- Develop stability demonstrating reverse phase HPLC test method for the determination of possible impurities in Testosterone cypionate
- Zorbax XDB-C8 (15 cm x 4.6 mm), 5 micron column was used
- Water was selected as solvent-A and Acetonitrile was preferred as solvent-B for mobile phase
- ICH guidelines was used to validate the method
- Forced degradation was performed
- The method is useful for routine quality control testing

About Authors

Amber Bharti completed his Master's Degree in science in 2003. He has around 18 years of experience in Analytical Research Development division in Major Pharmaceutical Companies. He is a doctoral student at Amity University, Uttar Pradesh, Noida-201303, India.

Dr. Ravindra Motiram Kumbhare received his PhD in Organic Chemistry from Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur (India) under the supervision of Dr V. N.Ingle. Presently he is working as Principal Scientist at CSIR-Indian Institute of Chemical Technology, Hyderabad (India). His research focused on process developments of various fluorochemicals, fluorinated heterocycles of biological interest, multicomponent reaction and there analytical technique. He guided 05 students for the award of PhD degree and 02 students are still working and also guided 20 MSc and B Pharmacy dissertation students. He published more than 35 research papers in national and international journal.

Dr. Christine Jeyaseelan is Associate Professor, Head of Department Chemistry at Amity Institute of Applied Sciences, Amity University, Noida. She has been awarded with doctoral degree by Nagpur University and is working in the field of electro-analytical and environmental chemistry, drug analysis and impurity studies. She has also written 2 books and has published several papers in national and international journals. She has 3 patents to her credit. She has more than 17 years of teaching experience.

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