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A Liquid Chromatography-UV (LC-UV) Method was Developed for Quantification of Six Potential Impurities in Androstanolone Active Pharmaceutical Ingredient

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Abstract

A sensitive, selective reverse phase method was developed for the quantitative determination of six potential impurities in Androstanolone active pharmaceutical ingredient. Efficient chromatographic separation was achieved on Zorbax Eclipse XDB C8 (250×4.6 mm, 5 µm) column with mobile phase containing a gradient mixture of solvent-A and solvent-B. The eluted compounds were monitored at 200 nm. All six potential impurities were identified by mass spectrophotometer and characterized by nuclear magnetic resonance. The developed method was validated as per ICH guidelines with respect to specificity, precision, linearity, quantitation limit, detection limit and accuracy. Regression coefficient value was greater than 0.99 for Androstanolone impurities. Detection limit of impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F were in the range of 0.0002-0.003% respectively. The quantitation limit of impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F were in the range of 0.003-0.013% respectively with respect to sample concentration. The accuracy of the method was established based on the recovery obtained between 92.72-106.90% for all impurities.

Keywords: Androstanalone; Validation; Impurity

Introduction

Dihydrotestosterone (commonly abbreviated to DHT) or 5α-dihydrotestosterone (5α-DHT), also known as androstanolone (5α-androstan-17β-ol-3-one) is a sex steroid and androgen hormone [1]. Androstanolone becomes physiologically active by binding to the androgen receptor, a member of the nuclear receptor super family that includes steroid hormone receptor and thyroid hormone receptors. Androstanalone has substantially greater affinity for the androgen receptor than does testosterone [2-4]. 5α-Dihydrotestosterone is the most potent endogenous androgen. Androstanalone is white color powder, molecular formulae is $C_{19}H_{30}O_2$ and its molecular mass 290.44 g/mol. Androstanalone is the most potent androgen and is responsible for the growth, development and maintenance of the normal secretary function of the prostate [5-9]. The most frequently exploited methods for a steroid analysis are gas-chromatography and high performance liquid chromatography, as reviewed recently [10]. While GC-MS has been generally applied for steroid analysis and their biological relevance, HPLC with UV absorption detection was applied to selected mixtures only [11]. Capillary electrophoresis is another method for the separation of steroid. Various modes, as micellar electro kinetic chromatography or micro emulsion electro kinetic chromatography was also reviewed [12-15]. The HPLC-MS methodology has been the relatively frequently used approach for steroid analysis such as HPLC-atmospheric pressure chemical ionization mass spectrometry [16] or HPLC- electrospary ionization (ESI) mass spectrometry. Numerous other reports deal with steroid analysis exists, which used GC, GC-MS and extraction methods [17-22]. A number of additional papers exist; however, they are dealing with the analysis of a single analyte. No methods have been reported in literature for the analysis of Androstanolone and its potential impurities in bulk drug samples. In this paper we developed and applied method for quantification of impurities by using HPLC-UV method. Hence, it was felt necessary to develop an accurate, rapid, selective and sensitive LC method for the determination of Androstanolone impurities. Objective of current study was to develop a significant method and also to carry out method validation of Androstanolone.

Chemical structures of Androstanolone and potential impurities are shown in figures 1-7.

Experimental

Materials and reagents

Samples of Androstanolone API and standards of impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F were obtained from Chemical Research and Development department, Troy Life sciences Bangalore. Deionized water was prepared using milli-Q plus water from Millipore (Bedford, USA). HPLC grade acetonitrile, analytical reagents orthophosphoric acid, triethyl amine, sodium hydroxide, were purchased from Merck India Limited (Mumbai, India).

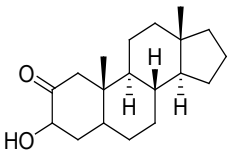

Figure 1: Structure and chemical name of Androstanolone, which is-17-hydroxy-10,13-dimethyl-1,2,4,5,6,7,8,9,11,12,14,15,16,17-tetradeca hydrocyclopenta [a] Phenonthren-3-one.

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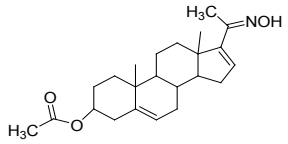
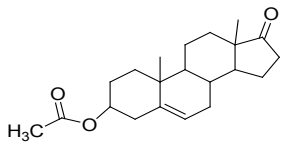


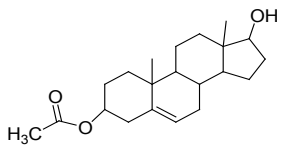
Impurity-A

Figure 2: Structure and chemical name of impurity-A, which is 17-(1-(hydroxyimino)ethyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl acetate.



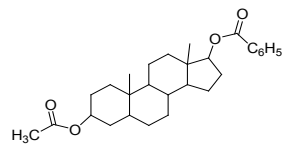
Impurity-B

Figure 3: Structure and chemical name of impurity-B, which is 10,13-dimethyl-17-oxo-2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-ylacetate.



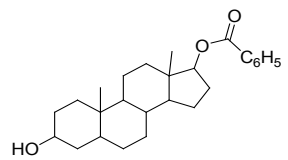
Impurity-C

Figure 4: Structure and chemical name of impurity-C, which is 17-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl-acetate.



Impurity-D

Figure 5: Structure and chemical name of impurity-D, which is 3-acetoxy-10, 13-dimethyl-hexadecahydro-1*H*-cyclopenta[a]phenanthren-17-yl-benzoate.



Impurity-E

Figure 6: Structure and chemical name of impurity-E, which is 3-hydroxy-10, 13-dimethyl-hexadecahydro-1*H*-cyclopenta[a]phenanthren-17-yl-benzoate.

Equipments and Chromatographic conditions

The HPLC system consisted of a Shimadzu prominence separate module LC-10ADVP equipped with dual wavelength absorbance and PDA detector water Empower-2 software was used for the data acquition and processing. Zorbax Eclipse XDB C (8) HPLC column of 250 mm×4.6 mm id, 5 µm particle size was used. The column was kept at 25°C. Mobile phase A consisted of 5.5 ml of orthophosphoric acid in 950 ml water, pH adjusted to 3.1 ± 0.05 with triethyl amine solution and Acetonitrile in the ratio (67:33), mobile phase B consisted acetonitrile in gradient mode (T_{min} A:B) T_0 70:30, T_2 70:30, T_{17} 30:70, T_{35} 30:70, T_{37} 70:30, T_{45} 70:30. The flow rate was set to 1.5 ml/min with detector wavelength fixed at 200 nm. The injection volume was 20 µl for a sample concentration of 7.5 mg/ml prepared in methanol.

Preparation of stock solutions for method validation

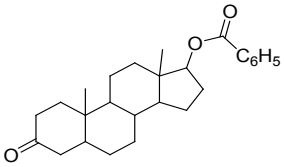
A test preparation of 7.5 mg/ml of Androstanolone API sample was prepared by dissolving in methanol. A stock solution of impurities was prepared by dissolving 10 mg each of impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F in 10 ml volumetric flask and made up with methanol. 7.5 ml of each individual stock solution was transferred into a 50 ml volumetric flask and made up to the volume with methanol. Again 2.5 ml of each individual stock solution was transferred into a 50 ml volumetric flask and made up to the volume with methanol. From this stock solution standard solution of 0.001 mg/ml of each impurity were prepared with respect to sample concentration (7.5 mg/ml). This standard solution was also used for checking solution stability and robustness parameters.

Results and Discussion

Method development

The preliminary investigation was conducted for the effect of various parameters of the method. The parameters assessed include the detection wavelength, the type and quantity of organic modifier, the column, the pH of mobile phase and column temperature. Androstanolone has UV absorption at 200 nm because the structure of Androstanalone is such that it does not have UV chromophore with significant absorbance. Hence, detection at 200 nm is selected for method development.

The method development attempts were made in different mobile phases. The first trial was carried out on an isocratic condition by using reversed phase C18 column (Inertsil ODS 250×4.0 mm) and a mixture of acetonitrile and buffer (0.1 M KH_2PO_4 pH 7.0) in the ratio 60:40 v/v. The system was not found suitable to elute all the impurities and separation of impurities. Furthermore, method development trials were performed in gradient mode. All the impurities and Androstanolone subjected to separation using gradient mode on different column like X-Terra, Phenomenex, Zorbax SB and Zorbax XDB and in different buffer like Acetate buffer and Phosphate buffer. Finally satisfactory peak shape and the resolution of closely eluting impurities were achieved on Zorbax eclipse XDB-C8 column (250×4.6 µm, 5 µ) by using phosphoric acid buffer with pH 3.1 adjusted by triethyl amine. Mobile phase-A is mixture of buffer and acetonitrile in the ratio (67:33 v/v) and mobile phase-B is acetonitrile. The flow rate of mobile phase was 1.5 ml/min. In the optimized conditions it was observed that Androstanolone, impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F were well separated with a good resolution. A typical



Impurity-F

Figure 7: Structure and chemical name of impurity-F, which is 10, 13-dimethyl-3-oxo-hexadecahydro-1*H*-cyclopenta[a]phenanthren-17-yl-benzoate.

retention time of impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F are 17.71 min, 13.11 min, 10.95 min, 31.92 min, 18.75 min and 20.89 min respectively. The optimized method was validated as per ICH guidelines.

Method validation

Validation study was carried out for the analysis of impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F. The system suitability and selectivity were checked by injecting 7.5 mg/ml of Androstanolone solution containing all impurities (Figure 8). Method validation results are summarized in table 1.

Accuracy and precision: Accuracy of the method was evaluated in triplicates at six concentration levels, i.e. from QL-150% of the analyte concentration (7.5 mg/ml). The percentage of recovery for each impurity was calculated at each level and found in the range of 92.72-106.90% (Table 1). The precision of the related substances method was checked by injecting six individual preparations of (7.5 mg/ml) Androstanolone with 0.1% of each impurity. Percentage RSD for peak areas of each impurity were calculated (Table 1). Precision was also determined by performing the same procedures on a different day. Impurity (1%) spiked solution of chromatograms have been depicted in figure 9.

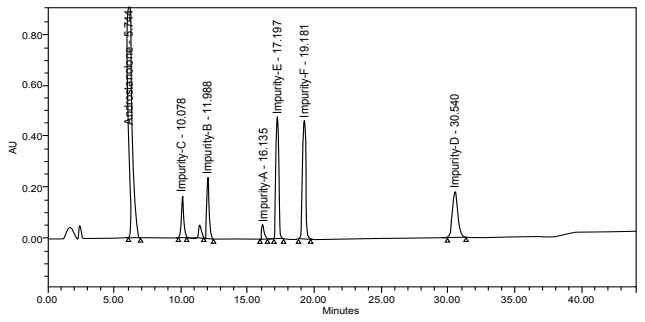


Figure 8: Chromatogram depicting Spiked solution of Androstanolone at 5.741 mins RT and its impurities, viz; impurity-C at 10.078 mins, impurity-B at 11.988 mins, impurity-A at 16.135 mins, impurity-E at 17.197 mins, impurity-F at 19.181 mins and impurity-D at 30.540 mins.

Parameter	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E	Impurity-F
System suitability						
RT	17.375	12.714	10.539	31.270	18.380	20.544
RRT	3.174	2.323	1.920	5.713	3.358	3.753
Linearity (r^2)	0.99876	0.99966	0.99937	0.99900	0.99969	0.99814
Quantitation						
Limit (%)	0.013	0.008	0.012	0.009	0.003	0.0011
Detection						
Limit (%)	0.003	0.002	0.003	0.0022	0.0007	0.0002
Precision at						
QL (RSD)	0.74	2.39	2.26	4.21	1.47	3.43
% Recovery at						
QL (n=3)	100.12	101.45	99.02	104.55	100.73	98.09
Accuracy (% Recovery)						
50%	100.61	99.11	103.14	98.62	101.05	97.86
75%	102.14	99.31	99.39	99.59	94.23	98.29
100%	97.25	100.18	99.90	98.48	101.23	103.42
125%	92.72	99.28	98.20	98.49	101.60	106.90
150%	100.66	98.44	98.83	99.68	98.44	105.80

Note: n, number of determinations; RT, retention time; RRT, relative retention time; R2, correlation coefficient. Table 1 shows different parameters of validation along with their respective results. It includes system suitability, linearity, quantitation limit, detection limit, precision at QL, and accuracy (with various concentrations such as 50%, 75%, 100%, 125%, 150%).

Table 1: Method validation summary report.

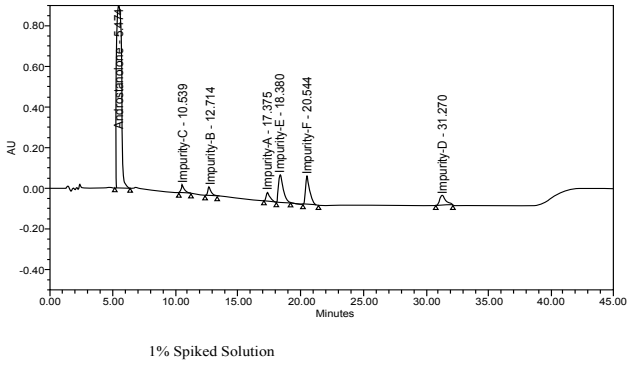


Figure 9: Showing the chromatogram of spiked solution of Androstanalone and its impurities, having concentration of 1% solution.

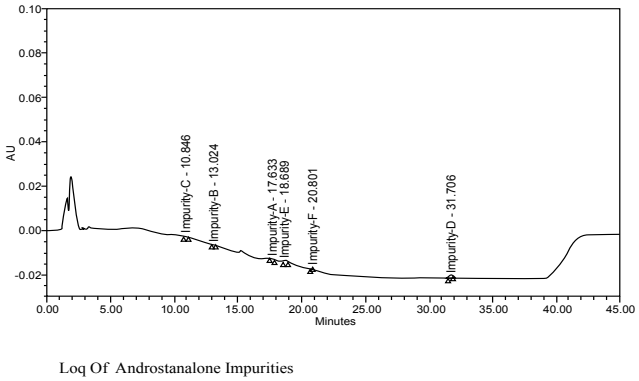


Figure 10: Chromatogram depicting the Quantitaion limit and their values of Androstanalone impurities, that is impurity-C=0.012, impurity-B=0.008, impurity-A=0.013, impurity-E=0.003, impurity-F=0.011, impurity-D=0.009.

Quantitation limit and detection limit: The quantitation limit and detection limit for all the impurities were determined by signal to noise ratio method. The QL for impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F were about 0.013, 0.008, 0.012, 0.009, 0.003 and 0.0011% respectively. The DL for impurity-A, impurity-B, impurity-C, impurity-D, impurity-E and impurity-F were about 0.003, 0.002, 0.003, 0.0022, 0.0007 and 0.0002% respectively, indicating high sensitivity of the method. QL solution of chromatograms has been depicted in figure 10.

Linearity and range: Linearity was established between ranges of QL to 0.2% of the analyte concentration (7.5 mg/ml). The correlation coefficient obtained was not less than 0.99 for all impurities. Standard deviation of peak area was significantly low and %RSD was below 2.0%.

Robustness and ruggedness: Close observation of analysis results of the deliberately changed in chromatographic conditions (flow rate, and column temperature) revealed that the resolution between impurity-A and impurity-E was greater than 2.0. The intermediate precision (ruggedness) of the method was also evaluated by a different analyst and different instrument in the same laboratory with %RSD areas of each impurity within 5.0. The experimental data are shown in table 2.

Validation Parameter	Resolution between Impurity-A and Impurity-E
Flow rate (ml/min)	
1.4	2.61
1.5	2.52
1.6	2.39
Column temperature (°C)	
25	2.54
30	2.65

Note: Changes by slight variation in flow rate and column temparature to ensure whether resolution between impurity-A and Impurity-E remains unaffected.

Table 2: Robustness of the LC method.

Solution stability: The solution stability of Androstanolone and its related substances was established by spiked sample solution. The solution stability experiments data confirmed that sample solutions were stable up to 48 hrs.

Conclusion

The newly developed LC-UV method for quantitative determination of Androstanolone and related substances was found to be sensitive, precise, accurate and specific. Thus, newly developed method has been validated as per regulatory requirements and can be used for routine and stability studies for the quantitative determination of potential impurities in Androstanolone drug substances.

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