Hygeia.J.D.Med. May 2016 - October 2016 ISSN 2229 3590
Hygeia:: journal for drugs and medicines
May 2016
Open Access www.hygeiajournal.com
Research article section: Pharmaceutical analysis
A Half Yearly Scientific, International, Open Access Journal for Drugs and Medicines
DOI:10.15254/H.J.D.Med.8.2016.154 Research Article

DEVELOPMENT AND VALIDATION OF A RP- UFLC METHOD FOR ESTIMATION OF SYNTHETIC STEROID HORMONE DANAZOL

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Keywords: Danazol, ICH, Validation, UFLC

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Received: 4 October 2015,
Revised: 24 December 2015
Accepted: 22 February 2016,
Available online: 15 June 2016

ABSTRACT

Plan: *Danazol is a synthetic steroid hormone with anti-estrogenic and anti-gonadotropic activities drug which acts on anterior pituitary suppressant by inhibiting the pituitary output of gonadotropins indicated for the treatment of endometriosis and benign breast disorders. The main objective of the research work was to develop simple, rapid and sensitive analytical method using RP-UFLC method for estimation of synthetic steroid hormone danazol in bulk and finished formulation.*

Methodology: *A simple, rapid and sensitive analytical method was developed. Separation of the chromatographic condition was done by using a mobile phase as isocratic flow with the concentration of 25 mM ammonium acetate (pH 4) and acetonitrile at the ratio of 50:50v/v. Flow rate of the mobile phase was maintained at l ml/min. Stationary phase used was C18 column (250 x 4.6 mm). Detection of danazol drug was carried out at 275 nm. The developed method was evaluated for the validation parameters such as specificity, sensitivity, linearity, accuracy, precision, ruggedness and robustness were performed as per the ICH guidelines.*

Outcome: *An optimized chromatographic condition for danazol drug was achieved and results showed good peak resolution. The developed method for the danazol can be used for the quantitative and qualitative estimation of bulk and finished formulations*

INTRODUCTION

A nonester synthetic hormone Danazol is gonadotropin inhibitor, structurally similar to testosterone or ethisterone and having weak androgenic effects. It is chemically known as 17α-Pregna-2, 4-diene-20-ynol (2, 3-D)-isoxazol-17-ol as shown in fig 1. It is used in the treatment of endometriosis, fibrocystic breast disease and hereditary angioedema[1]. It is used as doping agent to increase muscle strength, decrease healing time during injury, diminish fatigue and increased aggressiveness[2].

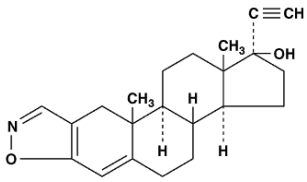

Fig 1. Structure of Danazol

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Hygeia journal for drugs and medicines, 2229 3590
Researcher id: D-8663-2014

US Pharmacopoeia has an official direct spectroscopic method for the assay of Danazol and HPLC (High Performance Liquid Chromatographic) method for the assay of Danazol capsules[3].

There are various methods reported for the Danazol determination either in formulations or in biological fluids. A quantitative TLC (Thin Layer Chromatography) method has been developed for Danazol analysis[4]. Various HPLC methods have been developed for Danazol analysis in bulk[5], formulations[6-10] and biological fluids[10-15]. Apart from HPLC methods gas chromatographic methods and immune assay methods[16] were also reported.

Even though analytical methods have been developed, a simple, rapid and sensitive method is essential for estimation of Danazol in bulk and pharmaceutical formulations. Hence we developed a simple, rapid, sensitive and economical RP (Reverse Phase)-UFLC[17, 18] (Ultra Fast Liquid Chromatography) method for the estimation of Danazol as per ICH guidelines [19].Morever this method also find advantage and economical when compared with existing method. The results obtained were promising.

MATERIALS AND METHODS

1.1. Materials

Standard were purchased from SDFCL, Mumbai. HPLC grade Acetonitrile (ACN), Methanol, ammonium acetate, triethyl amine and ortho phosphoric acid were obtained from SD Fine chemicals, Mumbai. Triple distilled water was obtained from Milli Q unit.

1.2. Identification studies

Danazol drug was accurately for 2 mg and kept in a capillary tube and placed in a melting point apparatus and the melting point was noted.

1.3. Solubility

The solubility of Danazol study was performed at various solvents such as methanol, acetonitrile and water respectively. About 10 mg was weighed in 25 ml a standard flask and the volume was made to 10 ml of different solvent mention above. The flasks were kept at 25±0.5^{0}C in isothermal shaker for 48 hours to reach equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 4000 rpm for 10−15min. The supernatant was taken and filtered through whatmann filter paper. The concentration of Danazol was optimized with suitable dilutions by using UV visible spectrometer.

1.4. Selection of wavelength for detection of components

From the above solution 100µg/ml of standard solution of Danazol were taken and the spectrum was recorded in the UV region from 200-400.

1.5. Preparation of standard stock solution

About 10 mg of drug was taken in 10ml standard flask. To that 5-6 ml of acetonitrile was added and kept in a sonicator for 15 min and the final volume was adjusted to produce 1000µg/ml which is kept in refrigerator for further use.

1.6. Preparation of Buffer (25mm)

Weigh about 0.9635 gm of Ammonium acetate was weighed accurately in a 500ml beaker. To that 200 ml of MilliQ water was added and kept in a sonicator for 10−15 min and the final volume was adjusted with MilliQ water and the pH was maintained to 4 by using ortho phosphoric acid.

1.7. Preparation of Calibration curve

From the stock solution, suitable dilutions were prepared using acetonitrile as solvent at the range of 10, 20, 40, 60, 80 and 100µg/ml respectively by measuring against the blank solution. The standard curve was plotted against concentration vs. peak area and the intercept and slope values were recorded. (Fig 2)

1.8. Chromatographic conditions

Shimadzu (Kyoto, Japan) UFLC system was equipped with binary gradient solvent delivery system pump (LC-20 AD), rheodyne injector (7725i) with 20 µL loop, and photo diode array (PDA) detector. LC solution software was used as a data station for interpreting the chromatograms. The separation of the analyties were carried out on Phenomenex C_{18} column (250 x 4.6 mm, i.d. 5µm) using ammonium acetate buffer (25mM, pH-4.0) and acetonitrile (50:50 v/v) as mobile phase at a flow rate of 1ml/min. The detection of the analytes were carried out at 275 nm.

1.9. Method validation

The developed method was validation as per the ICH guidance. The method was validated for specificity, selectivity, linearity, sensitivity, accuracy, precision, robustness and ruggedness.

1.10. Specificity and selectivity

Specificity of the developed method was carried out by comparing the standard and the sample retention time with six injections and the lack of interference peak were studied. The selected chromatographic condition should be ideal and should be free from impurities, matrix, degradants, excipients, preservatives and related impurities on selected standard retention time and peak.

1.11. Linearity and Sensitivity

Linearity was carried by plotting a calibration graph of analyte peak area (y axis) versus concentration (x axis) ranging from 10.0-100.0 µg/ mL as shown in table 1. Linear regression analysis was followed and the correlation of coefficient (r2) was used as measure of linearity. Limit of detection (LOD) and limit of quantitation (LOQ) were reported as 3 and 10 times the noise level obtained from three replicate injections of blank samples respectively.

1.12. Accuracy

Accuracy was evaluated to determine recovery of the standard solution form the sample solutions. Mean percentage (%) recovery of analytes was used as a measure of accuracy. Each sample concentration was spiked with the standard concentration and injected in triplicate and the percentage recovery was determined. Accuracy was evaluated to determine recovery of the standard solution form the sample solutions. Mean percentage (%) recovery of analytes was used as a measure of accuracy. (Fig 3 & Fig 4).

1.13. Precision and repeatability

Intermediate precision (intra- and interday) and repeatability were carried out on the analytes sample. Percentage relative standard deviation (% RSD) was considered as a measure of precision and repeatability. The analytes sample were prepared at three different concentration of low, middle and high concentration of linearity sample and injected six time on the same day and on three consecutive day for intra- and interday precision respectively.

1.14. Robustness and Ruggedness

The developed method was evaluated for robustness and ruggedness by slight changing the parameters such as instruments and operators, slight change in the optimized chromatographic conditions such as column temperature, flow rate and pH.

RESULTS & DISCUSSION

Optimization of the chromatographic condition was carried out based on the sharp peak, ideal separation, stability and repeatability of the standards. Numbers of trails were carried out to achieve the above criteria by changing the mobile phase parameters such as buffer concentration, buffer strength, organic concentration, pH ratio and standard phase such as different column as C8 and C18. Based on the trial and error method the developed method was optimized at the mobile phase concentration of 25mM of ammonium acetate and acetonitrile at the ratio of 50: 50 v/v with the pH of 4 respectively. Among the different solvent acetonitrile showed maximum solubility. Stationary phase used was C18 column (250 x 4.6 mm, i.d. 5µm). Detection of the optimized chromatograph was carried out at 275 nm. The developed method for danazol showed good separation at the retention time of 7.3 min respectively. Broadening of the peak was observed when decrease in the concentration of organic phase while shifting of peak was observed towards the void volume side due to the high solubility.

Similarly in aqueous phase when the concentration increase no peak was observed due to the low solubility where else decrease in the concentration of aqueous phase broadening of peak was observed. Change in the pH shows that peak splitting. Increase or decrease of the detection wavelength shows decrease in the response.

Specificity of the developed method was carried out by comparing the standard and the sample retention time with six injections and the lack of interference peak were studied. The selected chromatographic condition was free from impurities, matrix, degradants, excipients, preservatives and related impurities on selected standard retention time and peak. Hence the selected method was specific.

Linearity was carried by plotting a calibration graph of analyte peak area (y axis) versus concentration (x axis) ranging from 10.0-100.0 µg/mL was found to be linear in range.. Linear regression analysis was followed and the correlation of coefficient (r2) was used as measure of linearity. Regression equation for the linearity and the range was found to be 0.994 respectively. Accuracy was carried out by recovery studies by spiking the known standard concentration to the sample solution and found to be consistent at all the levels and the results are shown in Table 2.

Precision study was carried out at two different levels of intra- and interday levels. Six injection of three different concentration of low, middle and high concentration of linearity such as 20, 60 and 100 µg/mL were injected and the percentage RSD and standard deviation were calculated and results are well within the limits as shown in the Table 3 & 4.
The developed method showed no change in the chromatographic condition when evaluated for the robustness and ruggedness and found to be rugged and robust. Limit of detection and limit of quantification for danazol were studied base on the signal to noise ratio and found to be 50 ng/mL and 200ng/mL.

CONCLUSION

A simple, rapid, cost effective and sensitive method was developed for the danazol drug and validated as per the ICH guidelines such as specific, sensitive, accurate, precise, reproducible, robust, LOD and LOQ. The developed method for the danazol can be used for the quantitative and qualitative estimation of bulk and finished formulations.

Conflict of interest: None

ACKNOWLEDGEMENTS

Authors wish to thank Sree Vidyanikethan College of pharmacy, Tirupati for providing necessary facilities for carrying the research work.

Table 1. Calibration and linearity

S.No	*Concentration (μg/mL)*	*Peak Area*
1	10	1675
2	20	3327
3	40	6875
4	60	11200
5	80	13432
6	100	16876

Table 2. Accuracy studies

S.No	*Measured Concentration (μg/mL)*	*Actual Concentration (μg/mL)*	*% Nominal*
1	58.09	60	96.82
2	59.65	60	99.42
3	58.23	60	97.05
4	58.18	60	96.97
5	59.76	60	99.6
6	57.45	60	95.75
	Mean		97.60
	SD		1.55
	% RSD		1.59

Table 3. Precision studies: intra-day precision

	Concentration ((μg/mL)		
S.No	*20*	*60*	*100*
1	19.76	58.87	98.65
2	19.26	59.12	99.44
3	18.99	58.96	98.35
4	19.45	58.37	98.02
5	19.02	58.77	98.23
6	19.46	59.01	98.01
Mean	19.32	58.85	98.45
SD	0.29	0.26	0.54
%RSD	1.52	0.45	0.55

Table 4. Precision studies: inter-day precision

S.No	*Inter-day precision- 1 Concentration ((μg/mL)*			*Inter-day precision- 2 Concentration ((μg/mL)*		
	20	*60*	*100*	*20*	*60*	*100*
1	18.95	58.23	98.43	17.98	58.56	98.45
2	18.34	59.98	98.88	17.86	58.23	98.61
3	19.87	59.34	97.55	17.56	58.82	99.04
4	19.43	59.88	99.09	18.41	58.43	99.21
5	18.99	58.11	97.32	18.65	58.65	98.32
6	18.65	58.85	97.59	18.87	59.12	98.03
Mean	19.04	59.07	98.14	18.22	58.64	98.61
SD	0.55	0.80	0.76	0.50	0.31	0.45
%RSD	2.87	1.36	0.77	2.76	0.53	0.45

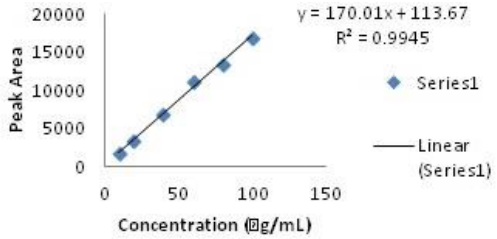

Fig.2. Calibration Curve

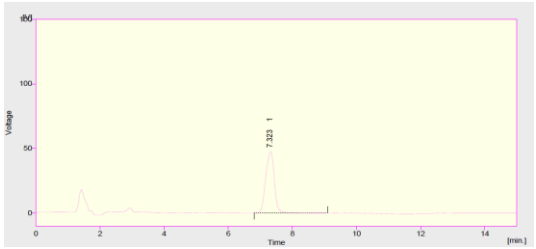

Fig.3. Typical HPLC chromatogram of Standard

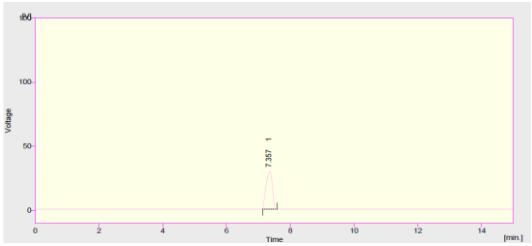

Fig.4. Typical HPLC chromatogram of Sample

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