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RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF STANZOLOL FOR ANALYSIS OF TABLETS DOSAGE FORM

M.V. Basaveswara Rao[1*], A. V. D. Nagendrakumar[2]

[1*]Department of Chemistry, Krishna University, Machilipatnam-521001, A. P, India
[2]Department of Chemistry, GITAM University, Visakhapatnam-530045, A. P, India

Corresponding Author

AVD. Nagendrakumar
Dept. of Chemistry,
Krishna University,
AP, India
Email: *Nagendra.git@gmail.com*

ABSTRACT

A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for rapid assay of Stanzolol in tablet dosage form. Isocratic elution at a flow rate of 1.0ml/min was employed on a symmetry C18 (250x4.6mm, 5µm in particle size) at ambient temperature. The mobile phase consisted of Methanol: water in the ratio of 90:10 v/v. The UV detection wavelength was 210 nm and 20µl sample was injected. The retention time for Stanzolol was 4.09min. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per the ICH guidelines. The method was successfully applied for routine analysis of Stanzolol in tablet dosage form.

Key words: Stanzolol, RP-HPLC, UV detection, recovery, precise, 210 nm

INTRODUCTION

Stanozolol is synthetic steroid commonly sold under the name Winstrol (oral) and Winstrol Depot (intramuscular). It was developed by Winthrop Laboratories in 1962. Stanozolol is a anabolic steroid derived from Testosterone. It is available in both Oral Tablet and aqueous suspension because it is not esterifies unlike the most injectable anabolic steroids. The drug has a high oral bioavailability, due to a C17 α-alkylation which allows the hormone to survive first-pass liver metabolism when ingested. Stanozolol has been used in both animal and human patients. In humans, it has been demonstrated to be successful in treating anaemia and hereditary angioedema. Angioedema causes episodes of swelling of the face, extremities, genitals, bowel wall and throat. Stanozolol may decrease the frequency and severity of these attacks. In Animals it is used to improve muscle growth, red blood cell production, increase bone density and stimulate the appetite of debilitated or weakened animals. Stanizolol is banned to use in sports competitions by International Association of Athletics Federations (IAAF) and many other sporting bodies because of its performance enhancing nature. It has been used in US horse racing.[1]

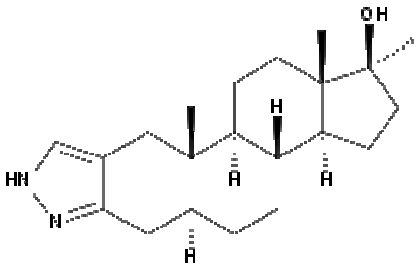


FIG.1 Stanozolol Structure

Systematic (IUPAC) name is 17β-Hydroxy-17-methyl-5α-androstano [3,2-c]pyrazole, $C_{21}H_{32}N_2O$, molecular weight is 328.49. The primary metabolites are unique to stanozolol and are detectable in the urine for up to 10 days after a single 5-10 mg oral dose. Methods for detection in urine specimens usually involve gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry.[2][3][4]

MATERIALS AND METHODS

Instrumentation: Peak HPLC containing LC-20AT pump and variable wavelength programmable UV-Visible detector and Rheodyne injector was employed for investigation. The chromatographic analysis was performed on a Chromosil C18 column (250 mm × 4.6 mm, 5µm). Degassing of the mobile phase

was done using a Loba ultrasonic bath sonicator. A Denwar Analytical balance was used for weighing the materials.

Chemicals and Solvents: The reference sample of Stanizolol (API) was obtained from Zydus. The Formulation (Neurabol) was purchased from the local market. Methanol, HPLC grade Water was used and purchased from Merck Specialties Private Limited, Mumbai, India.

The mobile phase: A mixture of Methanol: water in the ratio of 90:10 v/v was prepared and used as mobile phase.

Standard solution of the drug: For analysis 100ppm standard solution was prepared, required concentrations were obtained from 100ppm solution by appropriate dilution (40ppm).

Sample (tablet) solution: The formulation capsule of Neurabol (Stanozolol) was crushed to give finely powdered material. From the Powder prepared a 40ppm solution in mobile phase and then filtered through Ultipor N_{66} Nylon 66 membrane sample filter paper.

METHOD DEVELOPMENT

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wave length and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Detection wavelength: The spectrum of 10ppm solution of the Stanozolol in methanol was recorded separately on UV spectrophotometer. The spectra of Stanozolol were showed maximum absorbance at 210nm.

Choice of stationary phase: Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally the expected separation and peak shapes were obtained on chromosil C18 (250 mm x 4.6 mm, 5µm) column.

Selection of the mobile phase: In order to get sharp peak, low tailing factor and base line separation of the separation of the components, a number of experiments were carried out by varying the composition of various solvents and flow rate. To have an ideal separation of the drug under isocratic conditions, mixtures of solvents like methanol, water and Acetonitrile with or without different buffers indifferent combinations were tested as mobile phases on a Chromosil C18 column. A mixture of Methanol: water in the ratio of 90:10 v/v was proved to be the most suitable of all the combinations since the chromatographic peak obtained was better defined and resolved and almost free from tailing.

Flow rate: Flow rates of the mobile phase were changed from 0.5 – 1.5 mL/min for optimum separation. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution.

Optimized chromatographic conditions: Chromatographic conditions as optimized above were shown in Table.1 these optimized conditions were followed for the determination of Stanozolol in bulk samples and Formulations. The chromatogram of standard (4ppm) shown in Figure.2

Table.1 Optimized chromatographic conditions for estimation Stanozolol

Mobile phase	Methanol: water 90:10 v/v
Pump mode	Isocratic
Mobile phase P^{H}	7.5
Diluent	Mobile phase
Column	chromosil C18 column (250 mm x 4.6 mm, 5µ)
Column Temp	Ambient
Wavelength	210 nm
Injection Volume	20 µl
Flow rate	1.0 mL/min
Run time	8 min
Retention Time	4.093 min

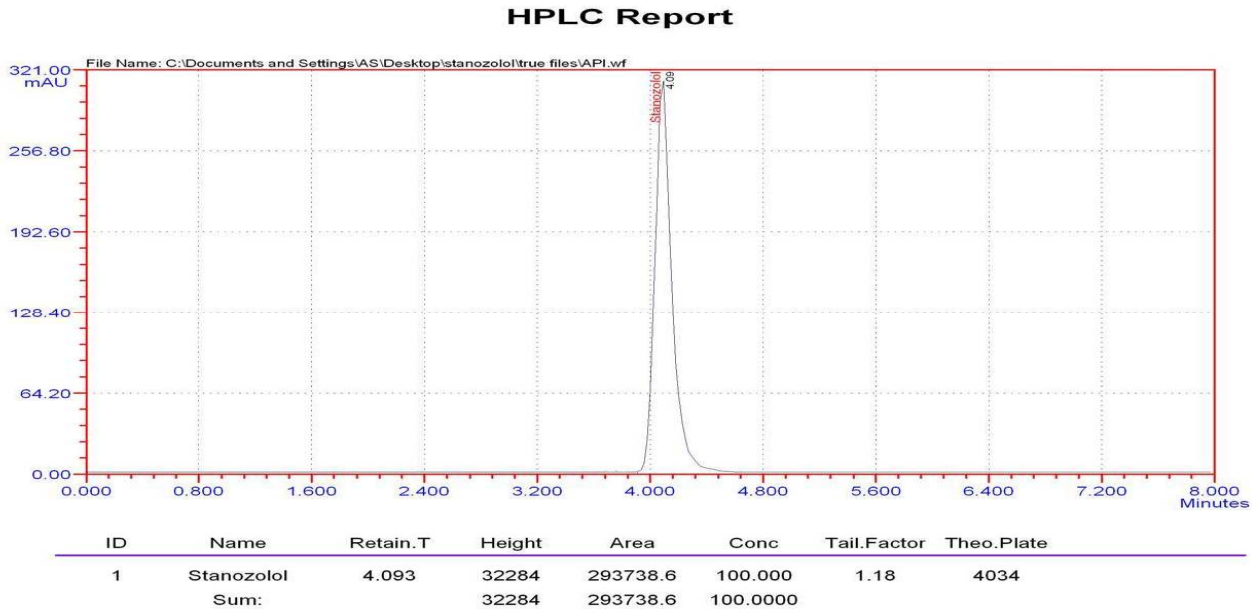


Figure 2: Chromatogram of standard solution

VALIDATION OF THE PROPOSED METHOD

The proposed method was validated as per ICH guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification and solution stability.

Specificity: The specificity of method was performed by comparing the chromatograms of blank, standard and sample (Prepared from Formulation). It was found that there is no interference due to

excipients in the tablet formulation and also found good correlation between the retention times of standard and sample. The specificity results are shown in Table.2

Table.2: Specificity study

NAME OF THE SOLUTION	Retention Time in Min
Blank	NO PEAKS
Stanozolol	4.093

Linearity: Linearity was performed by preparing mixed standard solutions of Stanozolol at different concentration levels including working concentration mentioned in experimental condition i.e. 4ppm. Twenty micro liters of each concentration was injected in duplicate into the HPLC system. The response was read at 210 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The regressions of the plots were computed by least square regression method. Linearity results were presented in Table.3 Linearity Chromatograms are given in Figure 4-9

Table.3: Linearity Results

Level	Linearity Level (std ppm is 40)	Mean peak area
Level -1	1	76755.0
Level -2	2	153636.1
Level -3	3	236354.8
Level -4	4	320987.1
Level -5	5	394189.0
Level-6	6	470558.5
	Slope Intercept Correlation coefficient	79294.52 2117.433 0.999

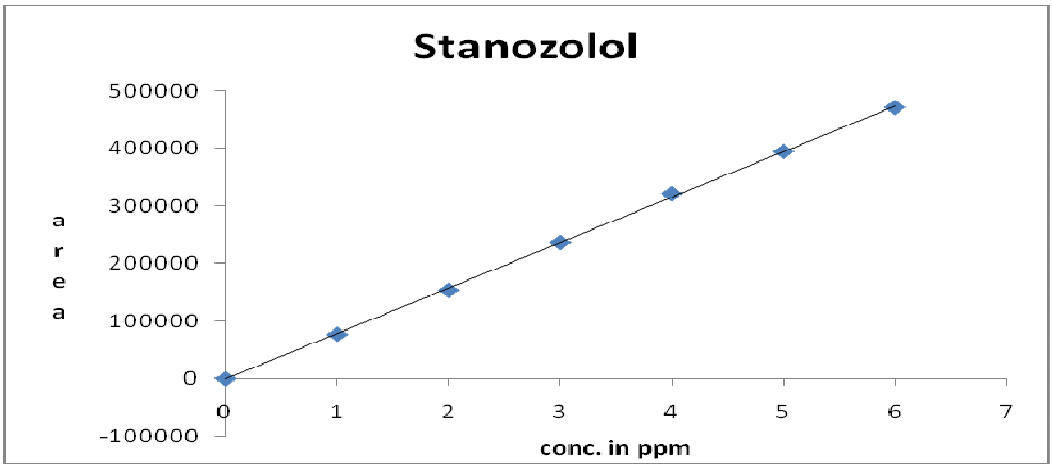
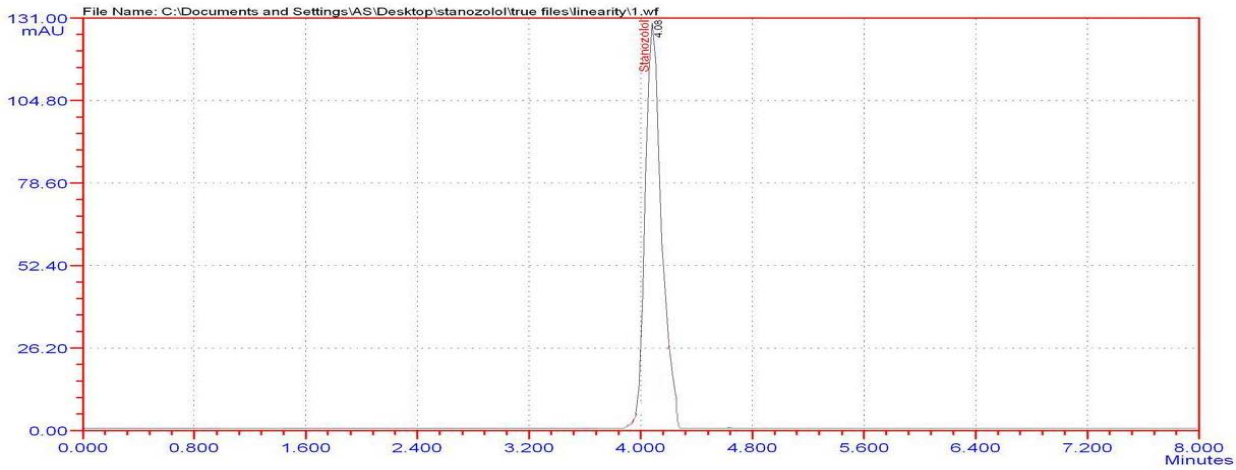


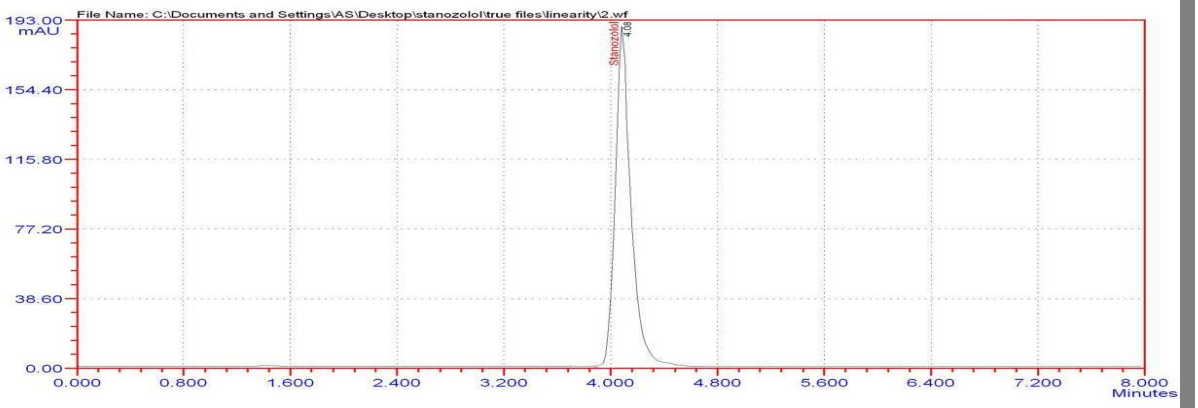
Figure .3: Linearity Graph

On X axis concentration of sample, On Y axis peak area response



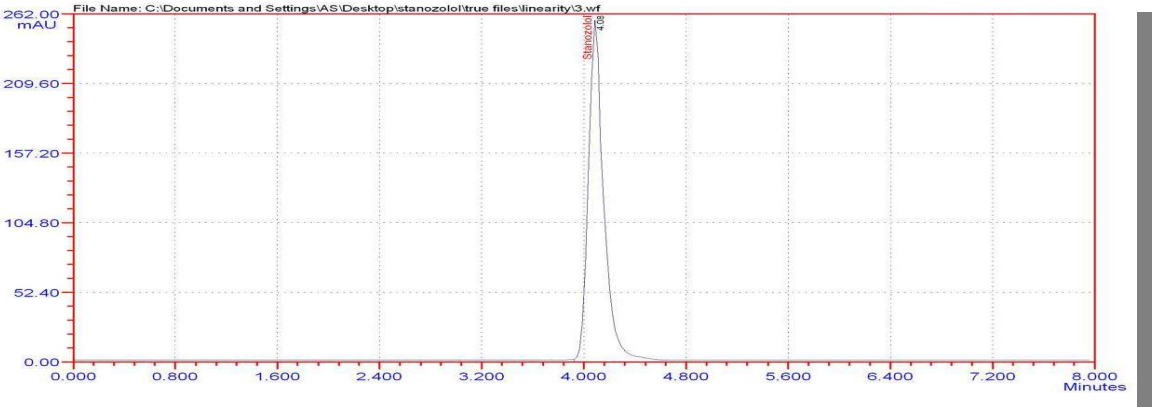
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Stanozolol	4.083	11350	76755.0	100.000	1.14	7267
	Sum:		11350	76755.0	100.0000		

Figue.4: Linearity level-1



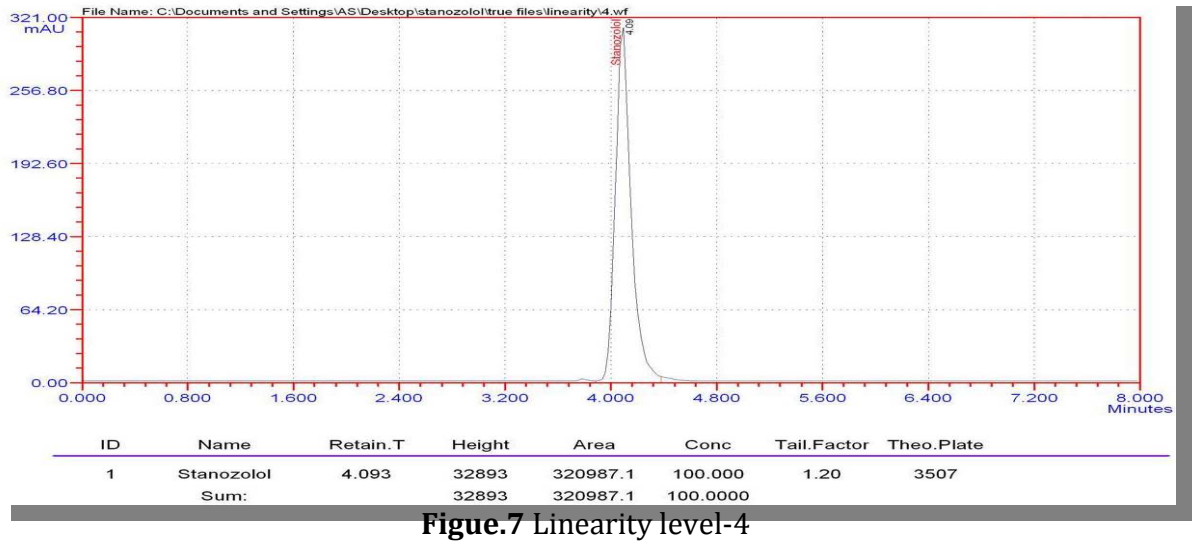
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Stanozolol	4.084	18890	153636.1	100.000	1.74	5024
	Sum:		18890	153636.1	100.0000		

Figue.5 Linearity level-2



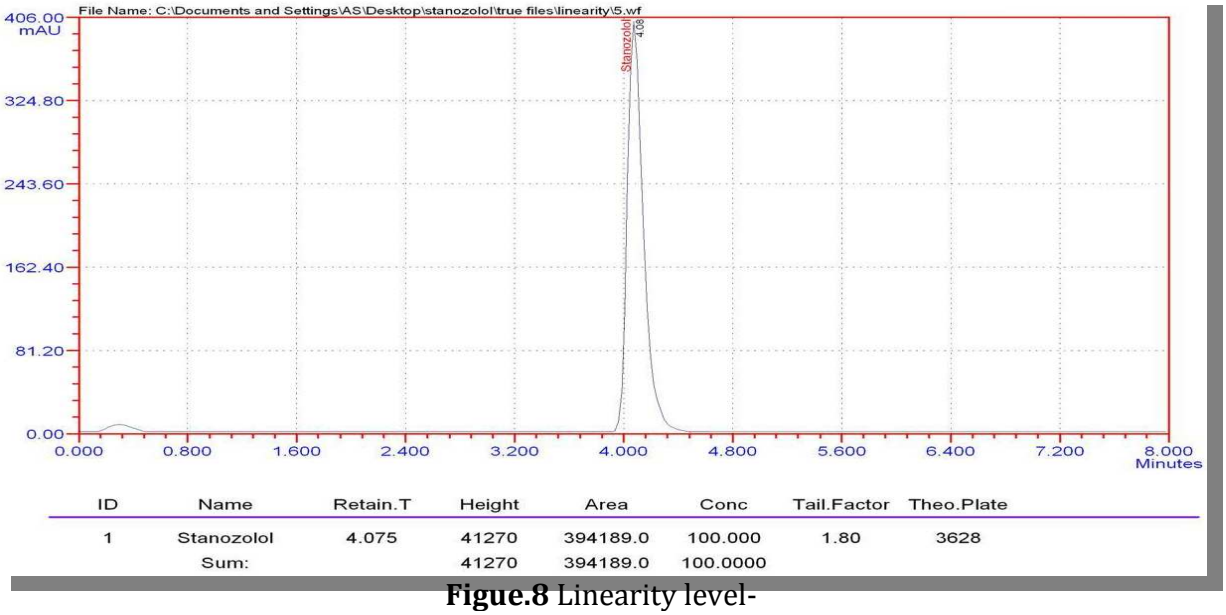
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Stanozolol	4.085	26626	236354.8	100.000	1.21	4221
	Sum:		26626	236354.8	100.0000		

Figue.6 Linearity level-3



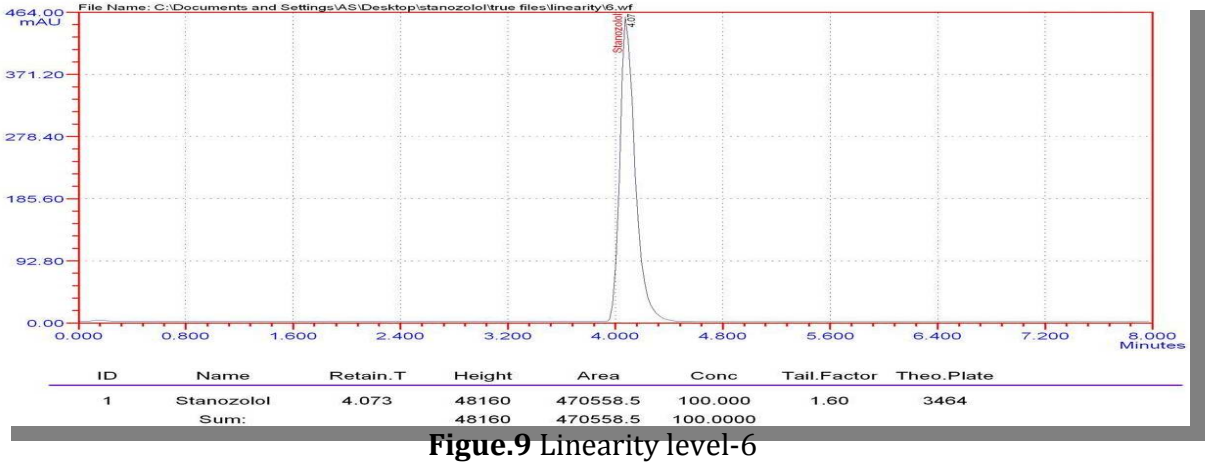
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Stanozolol	4.093	32893	320987.1	100.000	1.20	3507
	Sum:		32893	320987.1	100.0000		

Figue.7 Linearity level-4



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Stanozolol	4.075	41270	394189.0	100.000	1.80	3628
	Sum:		41270	394189.0	100.0000		

Figue.8 Linearity level-



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Stanozolol	4.073	48160	470558.5	100.000	1.60	3464
	Sum:		48160	470558.5	100.0000		

Figue.9 Linearity level-6

Precision: Precision is the degree of repeatability of an analytical method under normal Operational conditions. Precision of the method was performed as intraday precision, Inter day precision.

Intraday precision: To study the intraday precision, six replicate standard solutions (2ppm) of Stanozolol were injected. The percent relative standard deviation (% RSD) was calculated and it was found to be 0.465, which are well within the acceptable criteria of not more than 2.0. Results of system precision studies are shown in Table.4

Table .4: Precision Results

SAMPLE	CONC (PPM)	INJECTION NO	PEAKS AREA	R.S.D (Acceptance criteria ≤ 2.0%)
Stanozolol	2	1	149912.9	0.465
		2	148079.1	
		3	148995.4	
		4	146867.8	
		5	148878.6	
		6	149327.2	

Inter Day precision: To study the interday precision, six replicate standard solution of Stanozolol was injected on third day of sample preparation. The percent relative standard deviation (% RSD) was calculated and it was found to be 1.025, which are well within the acceptable criteria of not more than 2.0. Results of system precision studies are shown in Table 5

Table 5: Precision Results

SAMPLE	CONC (PPM)	INJECTION	PEAKS AREA	R.S.D (Acceptance criteria ≤2.0%)
Stanozolol	2	1	147396.2	1.025
		2	143862.5	
		3	149414.1	
		4	144817.7	
		5	147615.9	
		6	146102.9	

Accuracy: The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 50%, 100% and 150% level of 20ppm. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and % RSD was calculated

and results are presented in Table.6 Satisfactory recoveries ranging from 99.0 to 102.0 were obtained by the proposed method. This indicates that the proposed method was accurate.

Table.6: Accuracy Results

Level	% Recovery	R.S.D
50 %	99.33	0.191
	99.0	
	99.33	
100 %	99.5	0.383
	99.0	
	99.75	
150 %	99.83	0.120
	100.0	
	99.66	
	Mean % of Recovery 99.48	Mean R.S.D =0.248

Robustness: The robustness study was performed by slight modification in flow rate of Mobile phase, pH of the buffer and composition of the mobile phase. Stanozolol at 2 ppm concentration was analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature. The results of robustness study are shown in Table.7

Table.7: Robustness Results

Condition	Mean area	% assay	% difference
Unaltered	293738.6	100.0	0.0
Flow rate at 0.8 mL/min	292542.6	99.59	0.41
Flow rate at 1.2mL/min	292971.9	99.73	0.27
Mobile phase: MEOH: water			
92% 08%	294635.2	100.3	0.03
88% 12%	293245.4	99.83	0.17
pH of mobile phase at 7.3	294069.2	100.1	0.01
pH of mobile phase at 7.7	292896.7	99.71	0.29

System suitability: System suitability was studied under each validation parameters by injecting six replicates of the standard solution 2 ppm). The results obtained were within acceptable limits (Tailing factor ≤2 and Theoretical plates ≥2000) and are represented in Table.8. Thus, the system meets suitable criteria.

Table.8 System suitability results

Parameter	Tailing factor	Theoretical plates
Specificity study	1.18	4034
Linearity study	1.60	3646
Precision study	1.36	5378

Limit of detection and Limit of quantification: Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a detectable response. Limit of quantification (LOQ) is defined as the

lowest Concentration that can be quantified reliably with a specified level of accuracy and Precision. For this sample was dissolved by using Mobile Phase and injected until peak was diapered. After 0.05ppm dilution, Peak was not clearly observed. So it confirms that 0.05ppm is limit of Detection. For this study six replicates of the analyte at lowest concentration were Measured and quantified. The LOD and LOQ of Stanozolol are given in Table.9

Table.9: LOD and LOQ results

Parameter	Measured volume
Limit of Quantification	0.165ppm
Limit of Detection	0.05ppm

RESULTS AND DISCUSSION

To develop a precise, accurate and suitable RP- HPLC method for the simultaneous estimation of Stanozolol different mobile phases were tried and the proposed chromatographic conditions were found to be appropriate for the quantitative determination.Proper selection of the stationary phase depends up on the nature of the sample, and molecule Physico- chemical properties. Mixture of Methanol and Water (90:10 v/v) was selected as mobile phase and the effect of composition of mobile phase on the retention time of Stanozolol was thoroughly investigated. The concentration of the Water and methanol were optimized to give symmetric peak with short run time. A system suitability test was applied to representative chromatograms for various parameters. The results obtained were within acceptable limits and are represented in Table.1 Thus, the system meets suitable criteria.

Six points linear curve was constructed covering a concentration range of 1-6ppm (Three independent determinations were performed at each concentration). A linear relationship was observed between peak response and the concentration of Stanozolol. The linear regression equation was y = -1299 + 89210x (r= 0.999). The R.S.D. values of the slope were 79294.52 (n=3) and the R.S.D. of y-intercept was 2117.43 (n=3). Linearity values can shown in Table.3

Precision was evaluated by carrying out six independent sample preparation of a single lot of formulation. Sample was analyzed for five times after extracting the drug as mentioned in assay sample preparation of the experimental section. Low values of standard deviation denoted very good repeatability of the measurement. Thus it was showing that the equipment used for the study was correct and hence the developed analytical method is highly repetitive. RSD of intraday precision was found to 0.465. For the interday precision a study carried out on the same day on two consecutive days indicated a RSD of 1.025. This indicates good method precision. Results are shown in Table.4,5

The stability of Stanozolol in standard and sample solutions containing determined by storing the solutions at ambient temperature (20±10^{0}C). The solutions were checked in triplicate after three successive days of storage and the data were compared with freshly prepared samples. In each case, it could be noticed that solutions were stable for 48 hrs, as during this time the results did not decrease below 98%. This denotes that Stanozolol is stable and standard and sample solutions for at least 2 days at ambient temperature.

To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 50%, 100% and 150% to the proposed HPLC method. Results of recovery studies are shown in Table 4.7. The results showed good recoveries ranging from 99.0 to 101.45%. The mean recovery data obtained for each level as well as for all levels combined were within 2.0% of the label claim for the active substance with an R.S.D. < 2.0%, which satisfied the acceptance criteria set for the study.

The system suitability parameter like capacity factor, asymmetry factor, tailing factor and number of theoretical plates were also calculated. It was observed that all the values are within the limits (tailing factor ≤ 2and number of theoretical plates ≥ 2000).

The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and it could be used for the rapid and reliable determination of Stanozolol in tablet formulation.

CONCLUSION

The complete study results reveals that the developed and validated method has applicable for the determination of Stanozolol in pharmaceutical drug products. The developed method has applicable for regular quality control analysis.

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