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Development and Validation of Reversed Phase HPLC Method for Simultaneous Determination of Nystatin and Triamcinolone Acetonide in Synthetic Mixture

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PATEL ANKITA DIPAK *1, MADHURI HINGHE2

Department of Pharmaceutical Quality Assurance at ROFEL Shri G. M. Bilakhia College of Pharmacy, Vapi, Gujarat, India-396191

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ABSTRACT

A simple, rapid, precise and accurate high performance liquid chromatography method was developed for simultaneous estimation of Nystatin and Triamcinolone acetonide in Synthetic mixture. The separation was achieved using a Shim-pack solar C18 (250 × 4.6 mm, 5 m) column with a mobile phase of Acetonitrile: Methanol (50:50 % v/v). The flow rate was 1.0 ml/min, with UV detection at 240 nm. Nystatin and Triamcinolone acetonide had retention times of 6.89 and 3.35 minutes, respectively. Linearity for Nystatin and Triamcinolone acetonide was found to be in the range of 23-69 µg/ml and 1-3 µg/ml respectively. The research method was validated as per the ICH guidelines and the results were within the acceptance criteria for precision, linearity, specificity, and robustness.

INTRODUCTION:

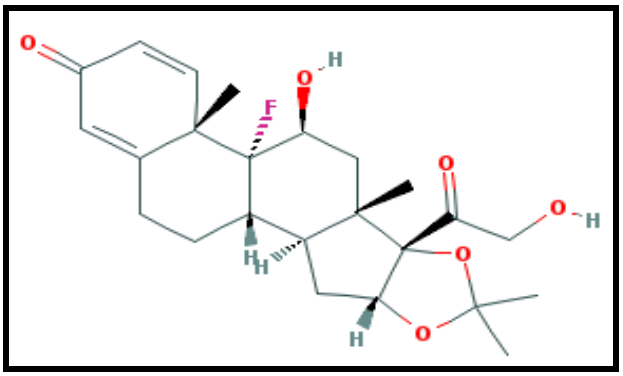

Figure No. 1: Structure of Nystatin

Nystatin (NYS) is chemically (1S,3R,4R,7R,9R,11R,15S,16R,17R,18S,19E,21E,25E,27E,29E,31E,33R,35S,36R,37S)-33-[(2R,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyloxan-2-yl]oxy1,3,4,7,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1] nonatriaconta 19,21,25,27,29,31-hexaene-36-carboxylic acid is an antifungal used to treat fungal skin infection or locally for superficial candidiasis. Nystatin is a channel-forming ionophore, its therapeutic effect via formation of a membrane-spanning pore in the fungal plasma membrane. Nystatin exerts its antifungal activity by binding to sterols in the fungal cell membrane. The membrane can no longer act as a selective barrier as a result of this binding, and potassium and other cellular contents are lost. The drug is not active against organisms (e.g., bacteria) that do not contain sterols in their cell membrane [3-6]. Nystatin is official in Indian Pharmacopoeia (IP) and British Pharmacopoeia (BP) and United States Pharmacopoeia (USP). IP and BP describe High Performance Liquid Chromatography (HPLC) method [7-9].

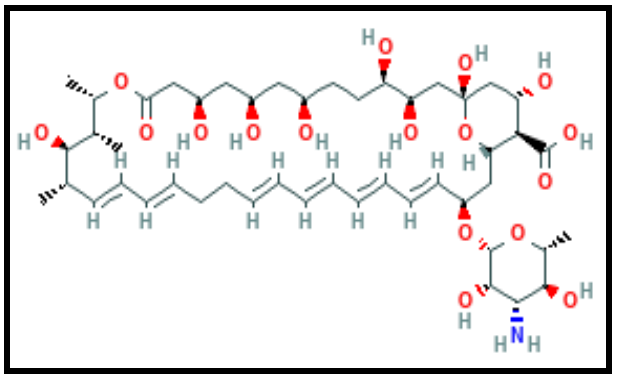

Figure No. 2: Structural of Triamcinolone

Triamcinolone acetonide (TCA) is chemically (1S,2S,4R,8S,9S,11S,12R,13S)-12-fluoro-11-hydroxy-8-(2-hydroxyacetyl)-6,6,9,13-tetramethyl-5,7 dioxapentacyclo [10.8.0.02,9.04,8.013,18] icosa-14,17-dien-16-one is a Corticosteroid. It is used to treat the inflammation caused by a number of conditions such as allergic reactions, eczema, and psoriasis. The dental paste form of triamcinolone is used to treat mouth ulcers. Triamcinolone acetonide binds to specific cytosolic glucocorticoid receptors and subsequently interacts with glucocorticoid receptor element on DNA and alter gene expression. This results in an introduction of the synthesis of certain anti-inflammatory proteins while inhibiting the synthesis of certain inflammatory mediators. Consequently, an overall reduction in chronic inflammation and autoimmune reactions are accomplished [10, 11]. Triamcinolone acetonide is official in Indian Pharmacopoeia (IP) and British Pharmacopoeia (BP) and United States Pharmacopoeia (USP). IP BP and USP describe High Performance Liquid Chromatography (HPLC) method [12-14].

Drug combination of the Nystatin and Triamcinolone acetonide used in treatment of certain fungus such as candida and help to relive the discomforted the infection, inflammation, itching and other skin conditions [16].

MATERIALS AND METHODS:

Chemicals, reagents, and samples:

Nystatin and Triamcinolone acetonide drugs substance were procured from Gift sample, S. Kant Healthcare Ltd., Vapi and Gift sample, ICPA Health Product1s Ltd., GIDC, Ankleshwar respectively. Acetonitrile was procured from (HPLC grade, Rankem, Maharashtra), Methanol (HPLC grade, Rankem, Maharashtra), Water (HPLC grade, Rankem, Maharashtra).

Instrumentation:

The HPLC system used was gradient HPLC Shimadzu LC-2010 CHT, series equipped with a 10 µL sample loop, and UV detector. The output signal was monitored and integrated using software LC solution version 1.25. Shim-pack solar C_{18} (250 × 4.6 mm, 5µm) column was used for the separation.

Chromatographic Conditions:

LC solution software was used to produce chromatographic separations using an HPLC (High-Performance Liquid Chromatograph) system with a SHIMADZU LC-2010 CHT with

UV Detector. As a mobile phase, Acetonitrile: Methanol (50:50 % v/v) was utilized. The experiment was conducted on a stainless steel Shim-pack solar column 250 mm long, 4.6 mm internal diameter loaded with porous silica particles of 5 mm diameter (Shim-pack solar C_{18}, 250mm×4.6mm, 5mm particle diameter column) kept at 40°C. A pump was in ingredient mode, and the mobile phase was flowing through the column at a rate of 1 ml/min. The runtime was 10 min. The analyte was measured at 240 nm and the injection volume was 10 µl. Peak -1 of Triamcinolone acetonide has a retention time of 3.35 minutes, while peak -2 of Nystatin has a retention period of 6.89 minutes.

Selection of Wavelength:

Aliquots of 2.3 ml from working solution of NYS (100 µg/ml) and 0.1 ml from working solution of TCA (100 µg/ml) were pipette out into two separate 10 ml of volumetric flask and volume was made upto the mark with methanol to get 23 µg/ml of NYS and 1 µg/ml of TCA. Each Solution of NYS and TCA was scanned between 200-400 nm using PDA Detector. Wavelength 240nm was selected from the overlain spectra of above solutions.

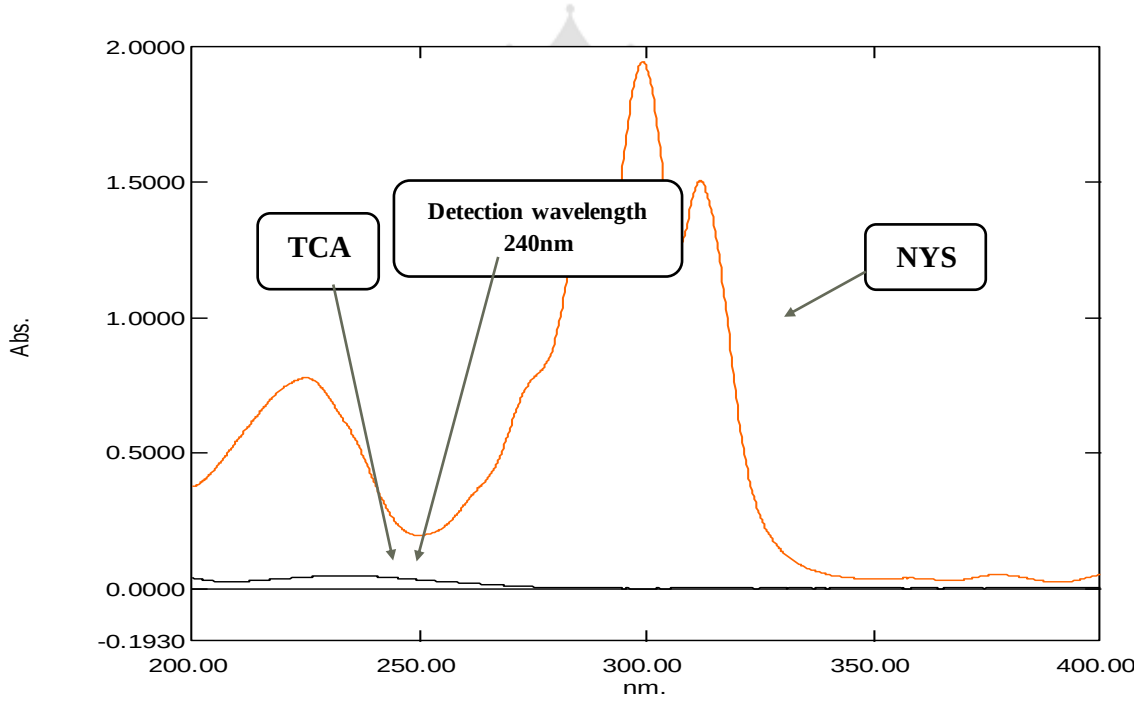

Figure No. 3: Overlain UV spectrum of NYS (23 µg/ml) and TCA (1 µg/ml) showing selection of wavelength detection

Preparation of solution:

1. Preparation of standard stock solutions:

An accurately weighed quantity of NYS (10 mg) and TCA (10 mg) were transferred to a separate 10 ml volumetric flask and methanol is added to both volumetric flasks. Volume was adjusted up to the mark with methanol to obtain standard solution having concentration of NYS (1000 µg/ml) and TCA (1000 µg/ml).

2. Preparation of Working stock solutions:

1 ml solution of NYS (1000 µg/ml) and TCA (1000 µg/ml) were transferred to a separate 10 ml volumetric flask and diluted up to concentration of NYS (100 µg/ml) and TCA (100 µg/ml) with mobile phase.

3. Preparation of Binary mixture of NYS and TCA:

Aliquots of 2.3 ml from working solution of NYS (100 µg/ml) and 0.1 ml from working solution of TCA (100 µg/ml) were taken into common volumetric flask and diluted up to 10 ml with mobile phase to make final concentration NYS (23 µg/ml) and TCA (1 µg/ml).

4. Preparation of synthetic mixture solution:

Weighed 1 gram of synthetic mixture [16, 17], Dissolve it in 50 ml of methanol and sonicate it for 15 min. Heat at 30 °C until base is dissolved and cool it at room temperature. Filter the extract through Whatman filter paper no. 42 and make up the volume up to 100 ml with methanol. Final stock solution containing NYS (23 µg/ml) + TCA (1 µg/ml).

METHOD VALIDATION:

System Suitability studies:

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

Specificity:

There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their synthetic mixture.

Accuracy study:

This experiment can be performed by the recovery test. Recovery of the method was evaluated at three different concentration levels by addition of known amounts of standard placebo preparation. For each concentration level, three sets were prepared and injected as duplicate. Accuracy of the method was determined by recovery studies. To the formulation (Preanalyzed sample), the reference standards of the drugs were added at the level of 0%, 80%, 100% , 120 %. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table 3.

Precision study:

Precision study was established by evaluating method precision and system precision study. Method precision of the analytical method was determined by analyzing six sets of the sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation and % RSD for the same was calculated. System precision of the analytical method was carried out to ensure that the analytical system was working properly. Standard solution was injected six times into system and chromatograms were recorded. (Table no. 5)

Limit of detection and Limit of quantitation study:

LOD is the lowest amount of the drug content which can be detected by the proposed method while LOQ is the lowest amount which can be quantified by the method. The guideline suggest minimum signal to noise ratio (S/N) more than 3.3 for LOD and more than 10 for LOQ. On the basis of linearity data theoretically, it can also be calculated by the given formula.

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ= residual standard deviation of regression line

S= slope of regression line.

Linearity:

Mixed standard solution of Nystatin and Triamcinolone acetonide were prepared with mobile phase in such a way that the final concentration of Nystatin and Triamcinolone acetonide is in the range of 23-69 µg/ml and 1-3 µg/ml for NYS and TCA respectively. The peak areas was recorded for all the peaks as shown in table-4 and figure no. 6, 7 for linearity of Nystatin and Triamcinolone acetonide. Figures 6, 7 indicate that the plots of peak area against concentration are linear, with regression coefficients of (R^2=0.9982) for Nystatin and (R^2=0.993) for Triamcinolone acetonide, demonstrating that the approach is highly linear over the working range.

Robustness study:

The robustness of method was determined to check the reliability of an analysis with respect to deliberate variation in method parameters. The typical variations are given below: Variation in mobile phase composition by ± 2 nm volume of solvent, variation in flow rate by ± 0.2 units, the robustness parameters for the method were shown in table 6 &7.

Assay:

Weighed 1 gram of synthetic mixture, dissolve it in 50 ml of methanol and sonicate it for 15 min. Heat at 30 ℃ until base is dissolved and cool it at room temperature. Filter the extract through whatmann filter paper no. 42 and make up the volume up to 100 ml with methanol. Final stock solution containing NYS (23 µg/ml) + TCA (1 µg/ml).

Chromatogram was recorded and the concentration of NYS and TCA was obtained by solving the regression equation.

RESULTS AND DISCUSSION:

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS:

Chromatographic analysis was performed on a SHIMADZU LC-2010 CHT with UV detector using LC solution software (C_{18}, 250 x 4.6 mm, 5µm column). Acetonitrile: Methanol (50:50 % v/v) was used as a mobile phase. The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 10 µl. Detection was performed at 240 nm.

Table No. 1: Optimized Chromatographic Conditions

Sr. No.	Parameters	Condition
1.	**Mobile Phase**	Acetonitrile: Methanol (50:50 v/v)
2.	**Flow rate**	1.0 mL/min
3.	**Run time**	10 min
4.	**Volume of injection**	10 µl
5.	**Detection Wavelength**	240 nm
6.	**Diluent**	Mobile Phase

VALIDATION:

Specificity:

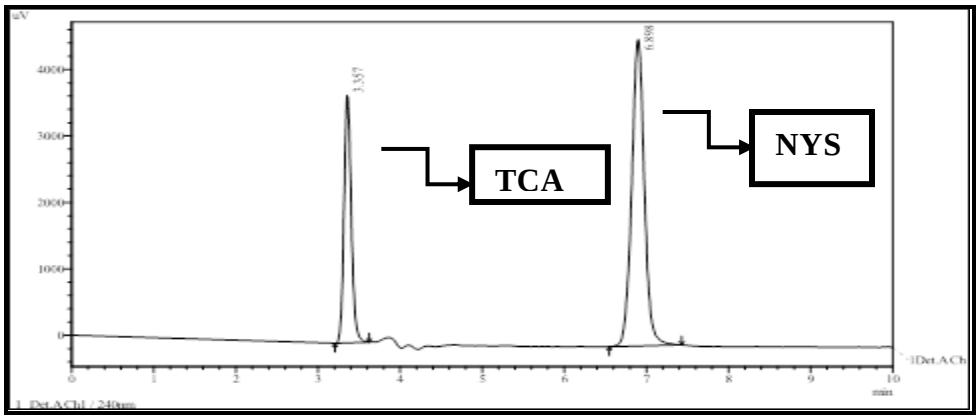

Figure No. 4: Chromatogram of sample NYS (23 µg/ml) + TCA (1 µg/ml)

System suitability:

The % RSD for the retention times and peak area of NYS and TCA were found to be less than 2 %. The plate count and tailing factor results were found to be satisfactory and are found to be within the limit.

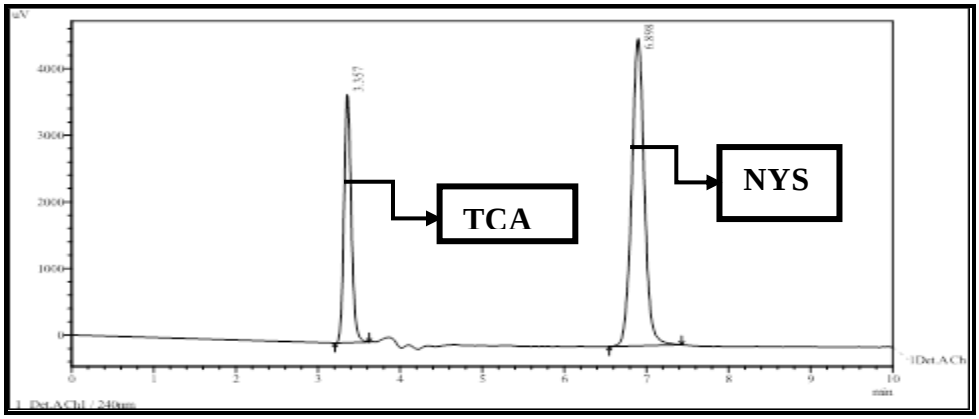

Figure No. 5: Chromatogram of sample preparation of NYS and TCA

Table No. 2: Results for system suitability of NYS and TCA

Drugs	Parameters	Mean ± S.D. (n=6)	% R.S.D.
NYS	Retention Time	6.8763 ± 0.0128	0.18729
	Theoretical Plate	62872.06 ± 84.9840	0.1351
	Tailing Factor	1.0253 ± 0.0023	0.2280
TCA	Retention Time	3.3475 ± 0.0124	0.3710
	Theoretical Plate	40446.77 ± 85.0490	0.2102
	Tailing Factor	1.1748 ± 0.0036	0.3065
Resolution		15.5466 ± 0.0320	0.2061

Accuracy:

For accuracy study data from nine determinations over three concentrations at 0%, 80%, 100% and 120% of expected sample concentration covering the specified range was determined & expressed as recovery values. The results were shown in table 3.

Table No. 3: Accuracy data for NYS and TCA

Drugs	Level	Amount of Sample (µg/mL)	Amount of Std. Spiked (µg/mL)	Total amount (µg/mL)	Amount of Sample found (µg/mL)	% Recovery
NYS	0%	23	0	23	22.71	98.78
	80%	23	18.4	41.4	40.69	98.29
	100%	23	23	46	45.98	99.96
	120%	23	27.6	50.6	50.45	99.72
TCA	0%	1	0	1	0.99	99.90
	80%	1	0.8	1.8	1.77	98.76
	100%	1	1	2	1.97	98.88
	120%	1	1.2	2.2	2.16	98.46

Linearity:

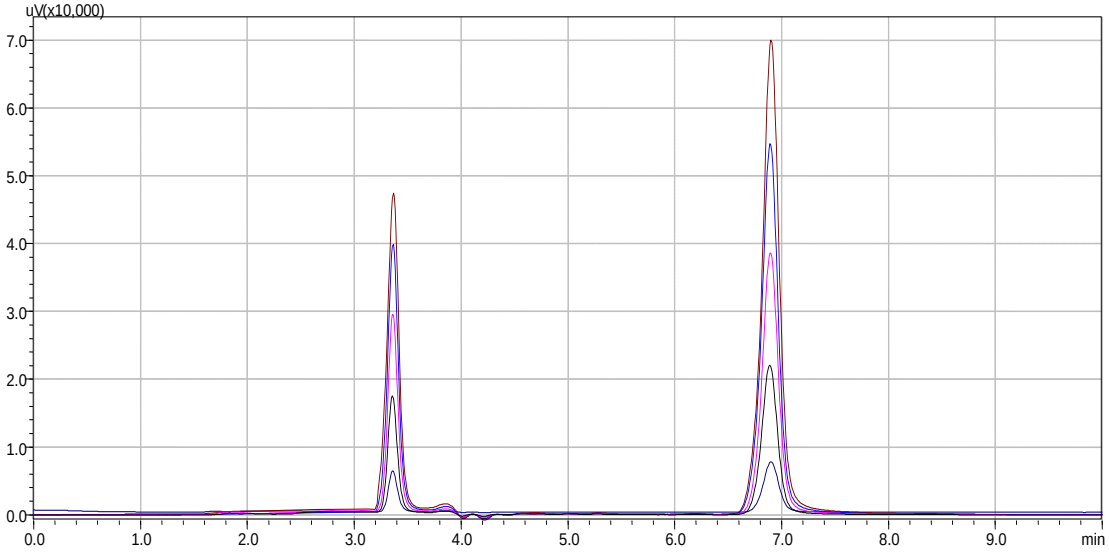

Figure No. 6: Overlain Chromatogram of NYS (23-69 µg/ml) and TCA (1-3 µg/ml)

Table No. 4: Linearity preparations of NYS and TCA

Sr. No.	NYS Concentration (µg/ml)	TCA Concentration (µg/ml)	NYS Mean Peak Area ± S.D. (n=5)	TCA Mean Peak Area ± S.D. (n=5)	NYS % R.S.D.	TCA % R.S.D.
1.	23	1	193263.0 ± 242.83	102743.4 ± 180.01	0.1256	0.1752
2.	34	1.5	367209.4± 750.94	190673.4 ± 497.81	0.2045	0.2610
3.	46	2	547023.0 ± 2826.12	267883.4 ± 412.05	0.5166	0.1538
4.	57	2.5	705436.8 ± 3176.27	366605.6 ± 1011.04	0.4550	0.2757
5.	69	3	856170.0 ± 2703.48	454304.8 ± 1279.74	0.3157	0.2816

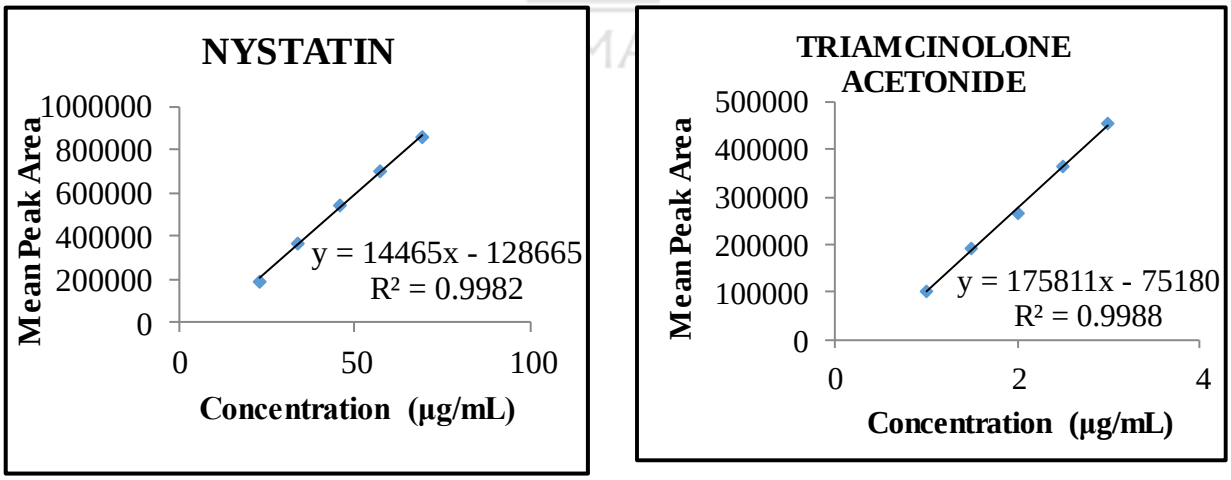

Figure No. 7: Calibration curve of NYS and TCA

Precision:

Table No. 5: Results for method precision of NYS and TCA

Parameter	Concentration (µg/ml)		Mean Peak Area ± S.D. (n = 3)		% R.S.D.	
	NYS	TCA	NYS	TCA	NYS	TCA
Repeatability (n=6) (% RSD)	46	2	548753 ± 1157.15	267913.7 ± 758.47	0.2108	0.2831
Intraday precision (n=3) (% RSD)	34	1.5	36763.66 ± 1084.93	190786.33 ± 756.12	0.2951	0.3963
	46	2	547069.66 ± 2371.35	268984.33 ± 923.52	0.4334	0.3433
	57	2.5	706710.66 ± 2497.08	367974.66 ± 1752.26	0.3533	0.4761
Interday precision (n=3) (% RSD)	34	1.5	367022.33 ± 1607.99	190740.00 ± 677.56	0.4381	0.3552
	46	2	548357.66 ± 2827.29	268043.00 ± 985.75	0.5155	0.3677
	57	2.5	704680.66 ± 4514.923	365823.33 ± 2147.41	0.6407	0.5870

LOD & LOQ:

The **LOD** for NYS and TCA was found to be 0.3299 µg/ml and 0.0257 µg/Ml respectively. The **LOQ** for NYS and TCA was found to be 0.9999 µg/ml and 0.0778 µg/ml respectively.

Robustness:

The result of robustness of the developed assay method was established in table no. 6, 7. The result shown that during all variance conditions assay value of the test preparation solution was not affected and it was the accordance with that of actual system suitability parameters were also found satisfactory. Hence the analytical method would be concluded as robust.

Table No. 6: Robustness data for NYS

Parameters	Level	Mean Peak Area ± S.D. (n=5)	% R.S.D.	Rt ± S.D. (n=5)	% R.S.D.
Mobile Phase (75:23:2 v/v)	**48:52 v/v**	198724.00 ± 459.02	0.2309	6.8666 ± 0.0245	0.3574
	52:48 v/v	199286.00 ± 600.36	0.3012	6.8630 ± 0.0286	0.4169
Flow rate (1.0 mL/min)	**0.8 mL/min**	197058.66 ± 933.50	0.4737	6.8553 ± 0.0330	0.4825
	1.2 mL/min	196660.66 ± 577.35	0.2935	6.8666 ± 6.8853	0.2155

Table No. 7: Robustness data for TCA

Parameters	Level	Mean Peak Area ± S.D. (n=5)	%R.S.D.	Rt ± S.D. (n=3)	%R.S.D.
Mobile phase (50:50 % v/v)	**48:52 v/v**	102945.66 ± 133.63	0.1298	3.3603 ± 0.0046	0.1375
	52:48 v/v	103032.66 ± 220.09	0.2136	3.3480 ± 0.0062	0.1865
Flow rate (1.0 mL/min)	**0.8 mL/min**	103387.33 ± 369.50	0.3573	3.3583 ± 0.0125	0.3723
	1.2 mL/min	102607.33 ± 461.88	0.4501	3.3273 ± 0.0058	0.1735

ANALYSIS OF SYNTHETIC MIXTURE:

Table No. 8: Analysis of Synthetic mixture

Synthetic mixture	Concentration (µg/ml)		Amount Obtain (µg/ml)		% Assay of NYS ± S.D (n=3)	% Assay of TCA ± S.D (n=3)
	NYS	TCA	NYS	TCA		
	23	1	22.87	0.9916	99.46 ± 0.0240	99.16 ± 0.0011

SUMMARY OF VALIDATION PARAMETER FOR PROPOSED METHOD

Table No. 9: Summary of RP-HPLC method

PARAMETERS	NYSTATIN	TRIAMCINOLONE ACETONIDE
Linearity (n=5)	23-69 µg/ml	1-3 µg/ml
Regression equation	y = 14465x - 128665	y = 175811x - 75180
Regression Co-efficient (R^2)	0.9982	0.9988
Correlation Coefficient (r)	0.9991	0.9993
Repeatability (n=6) (% RSD)	0.2108	0.2831
Intraday precision (n=3) (% RSD)	0.2951 - 0.4334	0.3433 - 0.4761
Interday precision (n=3) (% RSD)	0.4381 - 0.6407	0.3552 - 0.5870
LOD(µg/ml) (n=5)	0.3299	0.0257
LOQ(µg/ml) (n=5)	0.9999	0.0778
% Accuracy (n=3)	98.29 - 99.96	98.46 - 99.90

CONCLUSION:

Development and validation of RP-HPLC method for the estimation of Nystatin and Triamcinolone acetonide in synthetic mixture with the facilities and the results are incorporated in this thesis.

In conclusion, a validated RP-HPLC method has been developed for determination of Nystatin and Triamcinolone acetonide in synthetic mixture. The results show that the method was found to be specific, simple, accurate, precise and sensitive. The method was successfully applied for the determination of both drugs in synthetic mixture. In the future, this method may be applied for routine analysis of both the drugs in API and in synthetic mixture.

The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing % RSD less than 2. All

statistical data proves validity of the methods and can be used for routine analysis of synthetic mixture.

ACKNOWLEDGEMENTS:

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