



Fasten, simple, and specific stability of the avant-garde RP-HPLC method for estimation and validation of nystatin in pharmaceutical formulations

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ABSTRACT

In this study, a simple and reliable stability-indicating RP-HPLC method was developed and validated for the analysis of Nystatin in the pharmaceuticals. The chromatographic separation was performed in the isocratic mode on an Ion Pac column; Arcus EP-C18; 5 μ m, 4.6 \times 250 mm, 30 °C) using a mobile phase consisting of ammonium acetate 0.05 M buffer/ Methanol mixture (30:70) and a flow-rate of 1.0 mL/min with UV detection at 305 nm. The flow rate was set at 1.0 mL/min. The HPLC analysis method was validated in terms of linearity, precision, accuracy, specificity, and sensitivity, according to International Conference on Harmonization (ICH) guidelines. The results indicated that the retention time was 8 min, and no interferences were observed from the formulation excipients and stress degradation products. The specificity, linearity, precision, accuracy, LOD, and LOQ of the method were validated. The method was linear over the range of 5–500 μ g/mL with an acceptable correlation coefficient ($R^2 = 0.9996$). The method's limit of detection (LOD) and quantification (LOQ) were 0.01 and 0.025 μ g/mL, respectively. The results indicate that this validated method can be used as an alternative method for the assay of nystatin. This validated HPLC method could be used for routine analysis, quality control, and the stability of analysis of Nystatin formulations.



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INTRODUCTION

Nystatin is a fungicide belonging to the urine group that has a therapeutic effect on many fungi and

yeasts, including *Candida*. This compound exhibits toxic effects when administered intravenously while well absorbed through healthy skin or mucous membranes. It is also considered a safe drug when treating fungal oral and intestinal infections, Chemical formula: $C_{47}H_{75}NO_{17}$, Molecular mass: 926.1 g/mol (Ghari *et al.*, 2013; Cione *et al.*, 2010; Groll *et al.*, 1999).

Nystatin that has name According to AUPIC system (*Oxacyclooctatriaconta-6,8,12,14,16, 18-hexaene-23-carboxylic acid*) is an antifungal drug, which was developed at the beginning of the 50 years of the last century. Nystatin is effective for treating superficial infections caused by *Candida*. There are several forms of this drug, which is used to treat skin, mouth, pharynx, esophagus, and vaginal infections (Pons

et al., 1997; Nyst *et al.*, 1992). Since the body is unable to absorb nystamine from the gastrointestinal tract into the blood circulation, it is not used to treat systemic infections and cannot be injected. Side effects are rarely seen as a result of the use of the drug, and pregnant women can safely use it to treat vaginal infection caused by bleaching (Blomgren *et al.*, 1998). Figure 1 shows the chemical structure of Nystatin as follows,

Nystatin, as a polyene antifungal medication, is naturally derived from *Streptomyces noursei*. Many molds and yeast are sensitive to nystatin, and it has been used for the treatment of infections caused by *Candida*, *Cryptococcus*, *Histoplasma*, *Blastomyces*, and *Aspergillus spp.* It is now widely prescribed for fungal infections (Pappas *et al.*, 2009; Darsazan *et al.*, 2017; Shokraneh *et al.*, 2015). Nystatin could be safely administered orally as well as topically. According to current Pharmacopeia, including European and United States Pharmacopeia, nystatin, as well as many other antibiotics, is assayed by the bioassay method based on agar diffusion (Serri *et al.*, 2017).

The existence of several systems to express the potency of antibiotics leads to confusion, and moreover, the use of potency to express the content of bulk products can lead to difficulties in the interpretation of the content of pharmaceutical preparations. Although several HPLC methods can be found in the literature for Nys analysis to date, no stability-indicating-methods for NYS determination in drug products have been described. The aim of this study was to establish an analytical method which is specific and selective for Nys analysis in a commercial ointment product. This method was duly validated according to ANVISA regulations (Khoshkam and Afshar, 2014; Annapurna *et al.*, 2012; Sadeghi *et al.*, 2013; Ghari *et al.*, 2013).

Analytical method development and validation for newly introduced pharmaceuticals are of importance, as drug or drug combination may not be official in pharmacopoeia, and so analytical method for quantification is not available. To check and ensure the quality standards of drug molecules and their formulation, various analytical methods are employed. Most of the drugs in single or multi-component dosage forms can be analyzed by HPLC method because of the associated advantages like speed, greater sensitivity, improved resolution, specificity, accuracy, precision, reusable columns and ease of automation in this method (Chen *et al.*, 2006; Cumming *et al.*, 1990; Llabot *et al.*, 2007). This review article briefly discusses the RP-HPLC methods available for the estimation of antifungal agents

in bulk and in various formulations concentrating mainly on the mobile phase, stationary phase, and detector type.

The objective of the study

The objective of the study was to develop and verify the reverse phase-HPLC (RP-HPLC) method with a UV detector for the simultaneous determination of the NYS samples in the raw pharmaceuticals.

MATERIALS AND METHODS

HPLC-UV analysis

The LC-100 series S-HPLC features fully automatic digital computer control. Its electronic circuit design, internal mechanical structure design, processing technology, functions of cinematography workstation, and the technical criteria make it a leading instrument with excellent stability and reliability. Apparatus consisted of USA HPLC that classes LC series. The LC-100 equipped with a double-beam UV-visible spectrophotometer (Angstrom Advanced Inc., USA), model UV-100 PC with 1 cm path length quartz cell is used, and it is connected of IBM compatible computer.

The software UV-PC of personal spectroscopy software version Matlab, R2003b, was used of the proposed chemometric methods, and the partial least squares (PLS) was performed with PLS_Tool box for use with Matlab R2003b, VP pumps, and variable wavelength programmable UV detector. Peak areas were integrated using Angstrom Advanced Inc., an LC solution software program. The chromatographic separation and quantification were performed on the Ion Pac column; Arcus EP-C18; (250 mm × 4.6 mm; particle size 5 μm) analytical column at room temperature. The mobile phase was used as standard drug solutions. The tablet sample solutions were filtered through a millipore membrane filter before injection into the HPLC system.

Chemicals and reagents

Data and methodology

The ammonium acetate of the class HPLC grade was procured from Merck Ltd., Germany. The drug as a therapeutic effect on many fungi and yeasts were purchased from BORISOVSKIY ZAVOD MEDICINSKKH (Limited Belarus[®]). All other chemical reagents were of high analytical purity grade.

The standards material has the purity 98 %, according to manufacturer certificate, and were kindly donated by Limited Belarus[®] Drug Industries for medical devices and pharmaceuticals.

Configure the samples for measurement

1. HPLC grade solutions (Sigma-Aldrich® Chemie GmbH, Germany).
2. Stock standard solutions for Nystatin was prepared in the mixture from ammonium acetate and Methanol (30:70, v/v) as a buffer at pH 9.0 to prepare a concentration of 1 mg/ml from Nystatin.
3. Working standard solutions for NYS were prepared in a mixture from ammonium acetate and Methanol (30:70, v/v) a solvent to prepare the concentration (5.0, 10.0, 15.0, 20.0, and 25.0) $\mu\text{g/ml}$ for NYS.

Sample updating

To perform sample updating, the optimized PLS calibration set was augmented with different samples of Nystatin tablets® containing known amounts from standard NYS-500000 IU. NYSTATIN® tablets containing known amounts from standard NYS-500000 IU were manufactured by BORISOVSKIY ZAVOD MEDICINSKKH (Limited Belarus®). One known concentration to five unknown concentrations of samples containing different concentrations of each were added purpose for doing the initial calibration, and the predictive ability of the updated sample was checked using external validation samples. The standard additions method was used to calculate the unknown concentration of commercial pharmaceuticals where five standard concentrations were added to the concentration of the unknown of the sample.

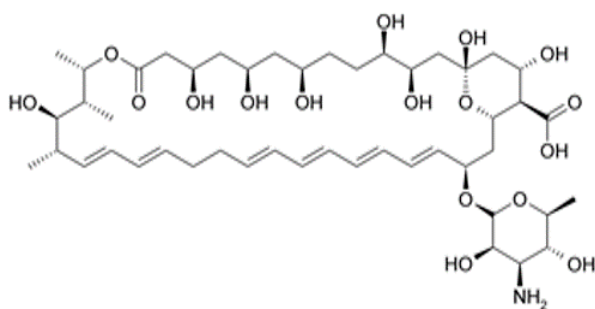


Figure 1: Chemical Structure of Nystatin

Reagents and Solutions

All reagents and solutions were prepared by deionized water, methanol, ammonium acetate, and acetic acid were purchased from Merck (Germany). Nystatin was purchased from Limited Belarus®. The standard material was purchased from Sigma-Aldrich (Germany).

Standard Solution

Nystatin stock solution (600 $\mu\text{g/mL}$) was prepared in mobile phase. This solution was then diluted

with the mobile phase in order to obtain the working standard solutions at concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 $\mu\text{g/mL}$. Fifteen $\mu\text{g/mL}$ of Nystatin (as an internal standard) was added into all of the working standard solutions, and the mixture was gently shaken.

Preparation of Sample Solutions

For the preparation of the sample solution, 10 tablets were taken and weighed individually. The average weight was calculated and finely powdered. An appropriate portion of this fine powder equivalent to 0.6 mg of nystatin was weighed and transferred to a 100 mL volumetric flask. This was dissolved and made up to the 100 mL with the mobile phase. The solution was sonicated for 10 minutes before filtration by a 0.45 μm membrane filter.

RESULTS AND DISCUSSION

Chromatographic Conditions

Table 1 shows the values of the basic parameters obtained using the RP chromatography system (RP-HPLC) of each of them were individually recorded for both Nystatin and in mixtures together in a tablet that show in Figure 2 A and Figure 2 B.

Assay of NYS in Pharmaceuticals

Five consecutive ($n = 5$) concentrations of 20 μl of the tablet solution (NYS 15.0 $\mu\text{g/ml}$) were injected into the HPLC system. Medicinal areas were determined at a 305 nm wavelength. The concentration of drugs in the tablets was determined either from the corresponding calibration curve or from the corresponding regression equation, Figure 3.

Assay of a validation set

The five laboratory mixtures (No. 5.0, 10.0, 15.0, 20.0, and 25.0) (Table 2) were selected to be used as external verification samples, and the procedure described in the calibration samples was followed. The concentrations of each component were calculated using the enhanced calibration sample PLS (Alsaad *et al.*, 2019; Abd-Alrassol *et al.*, 2019).

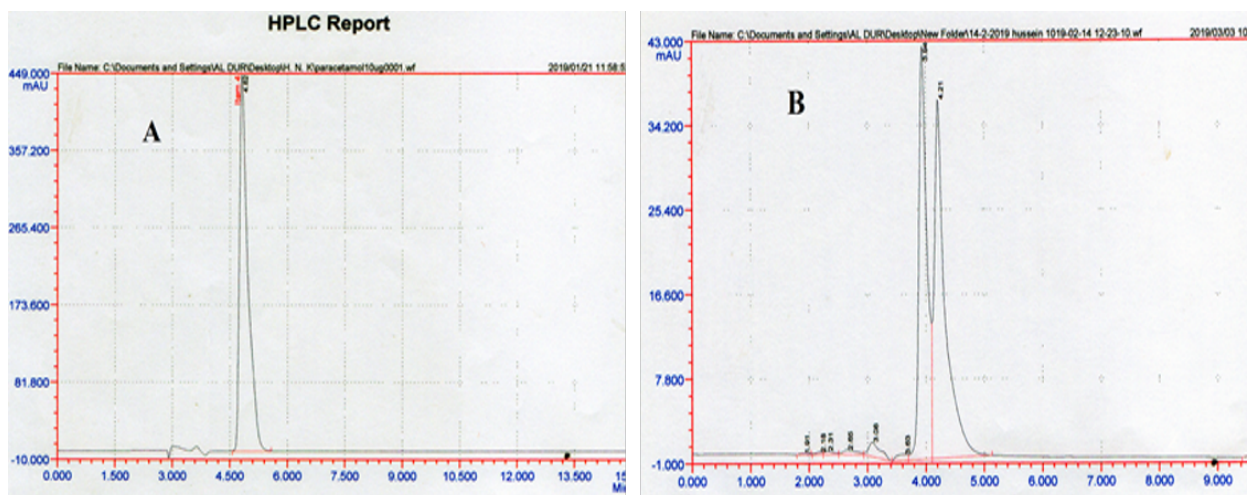
The concentrations of 100 mg and 50 mg were separately converted to 100 ml of the calibration flask, and 100 mL of methanol was added. The solution was left for 20 min, and the solution was then filtered from the precipitators and plankton. Appropriate dilution of solutions for the preparation of working solutions (100.0 $\mu\text{g/ml}$) of Nystatin concentration (15.0 $\mu\text{g/ml}$) was performed.

Stress degradation studies

Figure 4-A, B, C, D and E; Stress degradation studies were carried out using different ICH prescribed

Table 1: Parameters of RP-HPLC method

Column	Ion Pac RP-column; Arcus EP-C18; 5 μ m, 4.6 \times 250 mm)
Mobile phase	ammonium acetate 0.05 M buffer/ Methanol mixture (30:70) at pH 9.0
Flow rate	1.0 mL/min
Detection wavelength	At 305 nm
Column temperature	Room temperture
Injection volume	20 μ L
Run time	4.83 min

**Figure 2: Both Nystatin standard (A) and in mixtures together (B)**

stress conditions such as acidic, basic, oxidative, thermal, and photolytic stresses (Al-Salman, 2019b; Abd-Alrassol *et al.*, 2019) The melt cases of the degradation can be compared with the standard material in Figure 4.

Acid degradation

Figure 4-A; Tablets' powder equivalent to 15.0 mg of the NYS was taken in a 100 mL volumetric flask. 5 mL of 0.1 N HCl was added to the flask and kept at 80°C reflux conditions for 2–3 h. After completion of the stress, the solution was neutralized using 0.1 N NaOH and completed up to the mark with the mobile phase.

Base degradation

Figure 4-B; Tablets' powder equivalent to 15.0 mg of the NYS was taken in a 100 mL volumetric flask. 5 mL of 0.1 N NaOH was added in the flask and kept at 80°C reflux conditions for 2–3 h. After completion of the stress, the solution was neutralized using 0.1 N HCl and completed up to the mark with the mobile phase.

Oxidative degradation

Figure 4-C; Tablets' powder (equivalent to 15.0 mg of the NYS and 5 mL of 20% H₂O₂ was added in 100 mL volumetric flask. The flask was kept at 80°C

reflux conditions for 2–3 h. After completion of the stress, the flask was completed up to the mark with the mobile phase.

Thermal degradation

Figure 4-D; In this operation, tablets' powder (equivalent to 15 mg of the NYS was taken in glass Petri dish and placed in a hot air oven at 105°C for 2–3 h. After a specified time, the tablet powder was transferred to a 100 mL volumetric flask and made up to the mark with the mobile phase.

Photolytic degradation

Figure 4-E; For photolytic degradation study, the tablets' powder with equivalent to 15.0 μ g of the NYS was transferred into a glass Petri dish and placed in the direct sunlight for 2–3 h. After completion of the stress, the tablets' powder was transferred to a 100 mL volumetric flask and made up to the mark with the mobile phase.

Mean centering of ratio spectra method

Calibration curve

The standard calibration curves of the proposed method were prepared over concentration ranges of 5.0–25.0 μ g/mL. Each solution was prepared in triplicate, and 20 μ l of each solution was injected into the column. The calibration peaks were determined

at the wavelength of 305 nm. The calibration curves of the Nystatin was constructed by the relationship plotting of the peak area versus concentrations.

PLS sample to the calibration curve

Al-Salman (2019a); Abd-Alrassol *et al.* (2019) ,Figure 6-A, B, C, D, and E shows the use of a multi-design calibration curve for a number of concentrations for the purpose of verifying the estimation and measurement method. One concentrations (15 $\mu\text{g/ml}$) was used for sample and One concentrations (15 $\mu\text{g/ml}$) of a combination of Nystatin standard with a sample. Absorption spectra were studied within the Range wavelength at 295-315 nm.

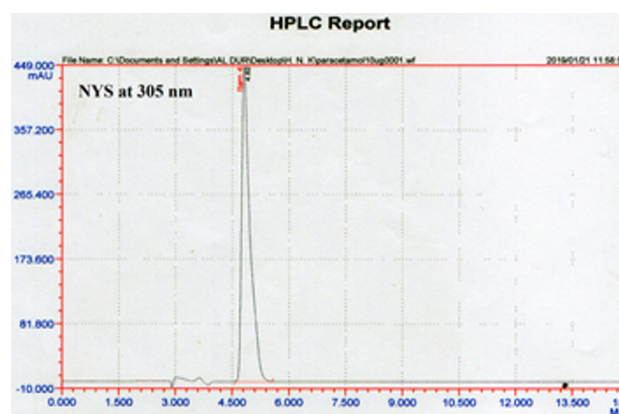


Figure 3: The corresponding calibration curve of NYS

The optimization of HPLC conditions

The chromatography conditions have been developed to separate all degradation products for Nystatin. During the RP-HPLC-UV method improvement, several experiments were performed using the Ion Pac Arcus EP-C18 column. 5 μm , 4.5 mm \times 250 mm, with appropriate mobile phase use consisting of ammonium acetate 0.05 M buffer/ Methanol mixture (30:70) at pH 9.0 with a flow rate of 1.0 ml/min. Wavelength was recorded at 305 nm. The retention time was recorded at 4.83 min. The form of good peaks was noted for the new analytical method (Figure 2 and Table 1).

The system suitability

A number of studies have been carried out for the purpose of adapting the RP-HPLC-UV system to the analysis of various Nystatin concentrations. The standard NYS (15.0 $\mu\text{g/ml}$) was used in five replicas (n = 5) of the same concentrations that were replicated using the optimal method. Table 3 shows the system's adequacy parameters. These results meet the requirements of the separation method for Nystatin estimates in various pharmaceuticals.

The validation of method and assay

Table 2: Concentrations of Nystatin in the calibration and validation sets

Sample Number	NYS $\mu\text{g/mL}$
1	6
2	6
3	4
4	8
5	9
6	10
7	12
8	10
9	11
10	13
11	6
12	11
13	14
14	20
15	21
16	19
17	24
18	25
19	23
20	24

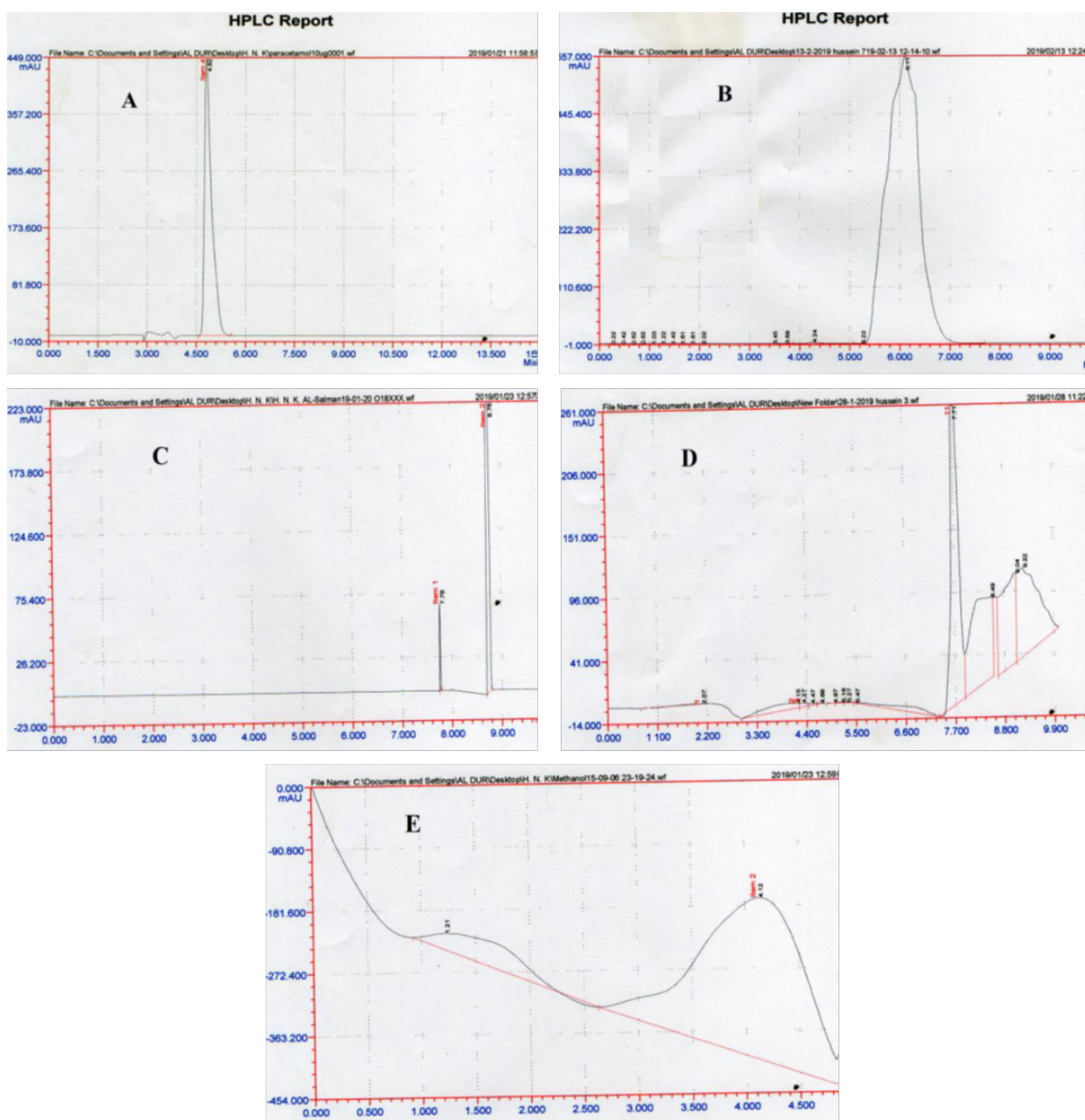
ICH (1996, 1994) In accordance with the ICH guidelines, the new chromatographic method HPLC-UV and the parameters such as specificity, linearity range, and sensitivity, regression, precision, accuracy, and rigidity were used to validate the method used. To assess the method validity, the effect of experimental conditions on the peaks areas for the analyzed materials was examined. The validity of the method was checked at a concentration of 15.0 $\mu\text{g/mL}$. Table 4 summarizes all the results. The results revealed that the peak areas for the drugs were small unaffected changes in flow rate, the composition of the mobile phase, temperature, and detection wavelength, indicating significant validity of the method.

Specificity of forced degradation samples

The specificity of the proposed method was studied using the study of forced degradation. The analysis was performed to ensure that the proposed method was able to separate Nystatin from the potential degradation products generated during the study of forced degradation. Studies were performed using acid, base, oxidation, photolysis, and heat for the tablet sample at a concentration of 15.0 $\mu\text{g/ml}$. Table 5 shows the results of forced decomposition. The highest percentage of deterioration occurred under the alkaline conditions of the drug.

Table 3: Parameters of the system suitability

Parameters	Value of the parameters	Recommended limits
Retention time	3.3 (%RSD 0.499)	RSD ≤ 1
Peak area	74543.6 (%RSD 0.432)	RSD ≤ 1
USP plate count	2071	~2000–2500
USP tailing factor	0.68	≤ 2 -2.5
Resolution	~ 0.23 min	≥ 2

**Figure 4: A, B, C, D and E: Stress degradation studies for NYS**

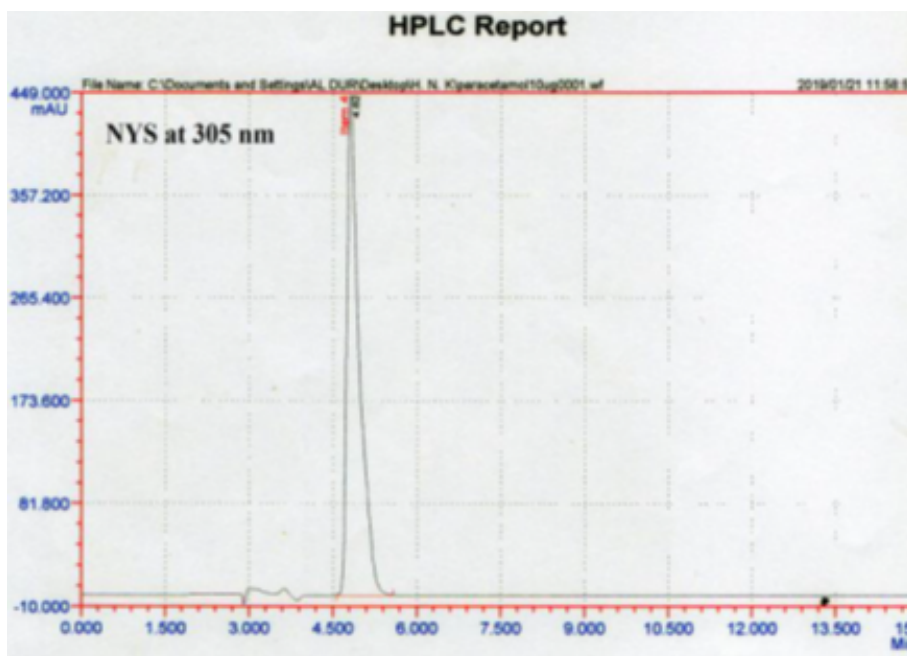


Figure 5: The calibration curves of the Nystatin

Table 4: Results of method robustness

Parameter	Robustness of NYS			
	Claimed Concentration ($\mu\text{g ml}^{-1}$)	Found ($\mu\text{g/mL}$)	% Recovery	%RSD
Column for NYS	15.0	15.0	100	0.312
System for NYS	15.0	15.0	100	0.353
Analyst NYS	15.0	14.6	97.3	0.365

Table 5: Results of forced degradation studies

Type of degradation	% Recovery	% of Degradation
Undegraded	100.12	0.000
Acid	97.551	1.314
Base	93.132	4.614
Oxidative	94.435	3.183
Photolytic	98.863	1.542
Thermal	96.727	1.123

Table 6: Regression statistics of the proposed method

Drug	R2	Standard error	Standard error estimate	Intercept	SLOPE	LLOD ($\mu\text{g/ml}$)	LLOQ ($\mu\text{g/ml}$)
NYS	0.9996	0.0227	0.0272	0.098x	0.4202	0.01	0.025

Table 7: Result of precision studies for NYS

Claimed concentration ($\mu\text{g/ml}$)	Interday		Intraday	
	Concentration	Mean \pm SD	Concentration	Mean \pm SD
5	5	100 \pm 0.313	5	100 \pm 0.312
10	10	100 \pm 0.344	10	100 \pm 0.344
15	15	100 \pm 0.353	14.8	98.6 \pm 0.311
20	19.88	99.4 \pm 0.315	19.7	98.5 \pm 0.355
25	24.90	99.6 \pm 0.367	24.6	98.4 \pm 0.355
15.0 $\mu\text{g/ml}$ Drug of NYS	14.8	98.6 \pm 0.355	14.8	98.6 \pm 0.377

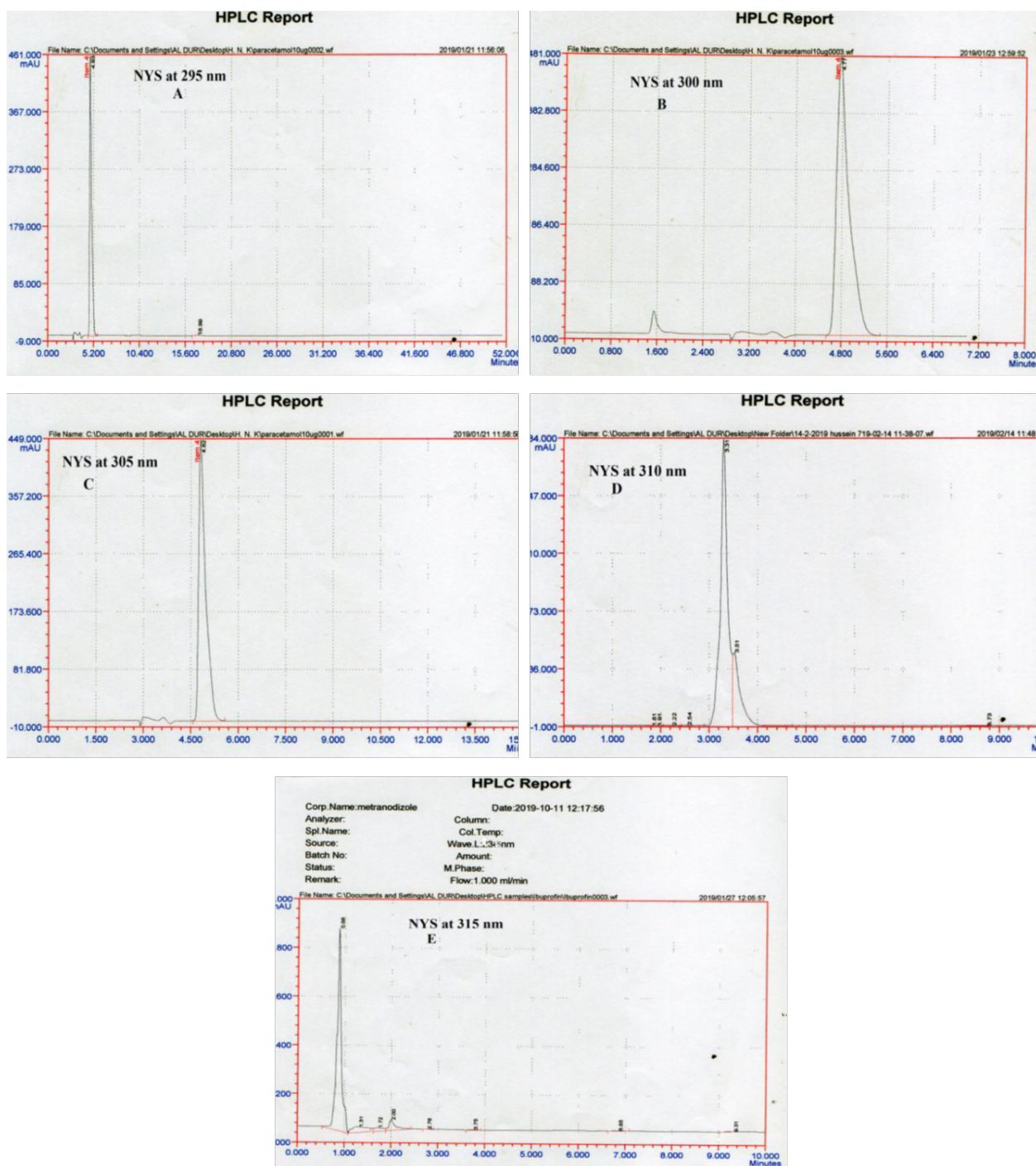


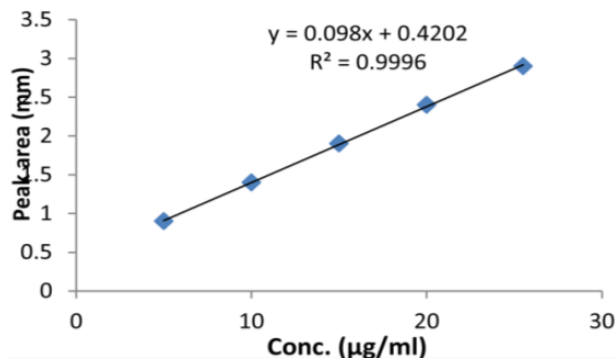
Figure 6: A, B, C, D and E: Partial least squares for a multi design calibration curve for standard calibration curve for NYS, except: Column, Ion Pac column; Arcus EP-C18; 5 μm , 4.6 mm \times 250 mm; wavelength 295-315 nm; a mobile phase consisted of ammonium acetate 0.05 M buffer/ Methanol mixture (30:70) at pH 9.0 with a flow rate of 1.0 mL/min, standard concentration: 15 $\mu\text{g/ml}$; injection volume: 20 μL

Table 8: Summarized results of accuracy

Parameters	NYS in standard	NYS in Nystatine drug
Claimed Concentration ($\mu\text{g mL}^{-1}$)	15.0	14.80
Found Concentration ($\mu\text{g mL}^{-1}$)	15.0	14.70
Recovery \pm RSD	100 \pm 0.365	98 \pm 0.315

Table 9: Assay of NYS in commercial tablets

Analyte	Labeled claim (mg)	Found (mg)	Mean (mg)	%Recovery	%RSD
NYS in drug (Limited Belarus®)	100	100	200	98.7	0.356

**Figure 7: Linearity of the calibration curve for NYS**

The lowest percentage of degradation for Nystatin occurred in the case of thermal and in the case of sunlight. One peak degradation was observed in decomposition products. Other degradation products due to stress do not interfere with the detection for Nystatin, so the method can be considered as an indicator of stability. The chromatograms are shown in Figure 4.

The linearity range and sensitivity

Ellepola *et al.* (2014) Under the optimum experimental conditions, a linear relationship was established by plotting the peaks areas for a drug against the drug concentration ($\mu\text{g/mL}$), that show in Figure 7. The concentration range was found to be 5.0–25.0 $\mu\text{g/mL}$. The linear regression analysis of the data gave from the following equations,

$$y = 0.098x + 0.4202 \quad (R^2 = 0.9996)$$

The Regression

Al-Shayyab *et al.* (2015) The sensitivity of the proposed method was assessed by calculating the limit of quantitation (LLOQ) and the limit of detection (LLOD). The LOD and LLOQ were calculated as follows,

$$LLOQ = 10 \times SD/S; \quad LLOD = 3.3 \times SD/S$$

Where SD= standard deviation of the drug response and S= slope of the calibration curve. LLOD values were found to be 0.0101 $\mu\text{g/ml}$, while LLOQ values were found to be 0.0251 $\mu\text{g/ml}$. These values demonstrate the satisfactory sensitivity of the proposed method for the analysis of the selected drug,

Table 6 shows the results of regression statistics of the proposed method.

Precision

Mima *et al.* (2012); Moshi *et al.* (1998) The precision was established by analyzing NYS at a concentration of 15.0 $\mu\text{g/ml}$. Two drugs (NYS-500000 IU were manufactured by BORISOVSKIY ZAVOD MEDICIN-SKKH (Limited Belarus®). was used to application analyzed Nystatin in pharmaceuticals. The system precision was tested by applying the developed method for the determination of Nystatin in the pure standard for 5 successive times ($n = 5$); after that, the application was used in the analyzes pharmaceutical. The method precision was tested by repeated analysis of Nystatin in tablet sample for 5 successive times ($n = 5$). The %RSD values for system precision, and method precision were $\leq 0.5\%$, indicating that the proposed method has good precision in the analysis of mixture Nystatin in pharmaceuticals. The results are summarized in Table 7.

Accuracy of the method

Sklenár *et al.* (2013); Wong *et al.* (2014); Melkoumov *et al.* (2013) For the pre-analysis tablet sample solutions, a known amount of standard solution was added at three different levels, 10%, 20%, and 30%. The solutions were reanalyzed by the proposed method. The results of the studies were the % recovery was between 99.88% and 100% with % relative standard deviation (RSD%) $\leq 0.3\%$. The results indicate good accuracy of the method. The selectivity of the method was demonstrated by the non-interferences of the excipients during an analysis of the Nystatin. The results are summarized in Table 8.

The Applications of the method

The analytical method of NYS in Pharmaceutical drugs were assessed by examining commercially available tablets (NYS- Belarus tablets® that claiming to contain 500000 IU). Table 9 summarizes the application results that indicate the values of % recovery and RSD%. The proposed method was accurate and precise in NYS analysis in dosages forms.

CONCLUSION

The presented review covers the analytical methods for the determination of antifungal agents in various pharmaceutical and biological samples alone or in combination with other drugs with the help of RP-HPLC. For the quantitative estimation of antifungal agents, the RP-HPLC method is the most common among others. All the reported methods are sensitive, precise, and accurate, consisting mainly of RP C18 column as stationary phase and variety of polar solvents (like methanol, water, and buffers) in different ratios as mobile phase. For the development of analytical methods, for newly developed or for upcoming novel antifungal agents, this can be taken for consideration.

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Author's contributions

This research was done individually in the laboratories of the College of Pharmacy, University of Basrah. This research was completed over a period of 3 months with serious and continuous work, and therefore, excellent results were obtained in finding an easy and sensitive method to estimate of determination of NYS in pharmaceutical formulations.

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