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Article

HPLC and Chemometric Methods for the Simultaneous Determination of Miconazole Nitrate and Nystatin

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High-performance liquid chromatography (HPLC) and chemometric methods were applied to the simultaneous determination of the two nonsteroidal antifungal drugs, miconazole (MIC) and nystatin (NYS). The applied chemometric techniques are multivariate methods including classical least squares, principal component regression and partial least squares methods. The ultraviolet (UV) absorption spectra of the standard solutions of the training and validation sets in methanol are recorded in the range of 280–320 nm at 0.2-nm intervals. The HPLC method depends on reversed-phase separation using a C18 column. The mobile phase consists of a mixture of methanol–acetonitrile–ammonium acetate buffer (pH 6; 50 mM) (60:30:10 v/v/v). The UV detector was set at 230 nm. The developed methods were validated and successfully applied to the simultaneous determination of MIC and NYS in their tablets. The assay results obtained using the chemometric methods were statistically compared to those of the HPLC method and good agreement was observed.

Introduction

Miconazole nitrate [l-(2,4-dichloro-β-((2,4-chlorobenzyl)oxy) phenethyl)imidazole] is a synthetic imidazole derivative, applied widely as the nitrate salt (MIC) with broad-spectrum antifungal activity (1, 2). It has been established as a useful drug for the treatment of various systemic mycoses. It is also active against Gram-positive bacteria. Nystatin (NYS) is a polyene antifungal antibiotic that is of particular interest because it exhibits remarkable action against a wide range of pathogenic and non-pathogenic yeast and fungi. It is also used for the prophylaxis and treatment of candidiasis of the skin and mucous membranes. For the treatment of oral candidiasis, NYS is administrated in either a suspension or suppositories (100,000 IU = 20.5 mg) (3, 4). MIC is formulated with NYS as vaginal suppositories for the treatment of vaginal or vulvovaginal candidiasis (moniliasis), vaginitis or vulvovaginitis caused by other sensitive fungi or Gram-positive bacteria and vaginal mycosis secondarily infected by Gram-positive bacteria. Several analytical methods have been reported in the literature describing the determination of miconazole nitrate (5–9) or nystatin (10–14) alone or in combination with other drugs. Neither high-performance liquid chromatography (HPLC) nor chemometric spectrophotometric methods have yet been reported for the simultaneous determination of MIC and NYS in their pharmaceutical formulations.

In this study, chemometric spectrophotometric and HPLC methods have been developed for the simultaneous determination of MIC and NYS in their synthetic mixtures and vaginal suppositories. The studied chemometric spectrophotometric methods are multivariate methods including classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS).

The primary advantages of these techniques are the higher speed of processing data concerning the values of concentration and absorbance of compounds with strongly overlapping spectra. Additionally, the errors of calibration models are minimized by measuring the absorbance values at many points in the wavelength range of the zero order spectra.

CLS, sometimes known as K-matrix calibration, is so called because it originally involved the application of multiple linear regression (MLR) to the classical expression of the Beer-Lambert law of spectroscopy. PCR combines the principal component analysis (PCA) spectral decomposition with an inverse least square regression (ILS) to create a quantitative model for complex samples. The eigenvectors resulting from data decomposition represent the spectral variations that are common to all of the spectroscopic calibration data. PLS is closely related to PCR, but in PLS the concentration data matrix is also used in the spectral decomposition. Both PCR and PLC produce robust models. They remove noise from the absorbance and concentration data.

Experimental

Instrumentation

A double-beam Shimadzu (Kyoto, Japan) ultraviolet visible (UV–Vis) spectrophotometer, model UV-1601 PC equipped with 1-cm quartz cells and connected to an IBM-compatible computer. The bundled software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth was 2 nm and the wavelength scanning speed was 2,800 nm/min. CLS, PCR and PLS analyses were conducted using the Chemometrics toolbox 2.1 software (15) for use with MATLAB 7.10.

Liquid chromatography was performed on a Knauer (HP) liquid chromatographic system (Berlin, Germany) equipped with an A54103 smart line pump 100, an HP variable UV detector 2500, and an A1357 manual injection valve with 20 μL sample loop. Eurochrom for Windows Basic Edition V3.05 was employed for data collecting and processing. A Phenomenex Luna (Germany) C18 column (250 × 4.6 mm i.d., 5 μm ps) was used for the separation. The detector was set at λ = 230 nm.

Materials and reagents

MIC and NYS were supplied by Medical Union Pharmaceuticals (MUP) (Ismailia City, Egypt) and certified to contain 99.8 and 99.9%, respectively.

Commercial Monicure Plus vaginal suppositories (batch no. 7361056) (Pharaonia Pharmaceuticals, Alexandria City, Egypt) were used. Each vaginal suppository was labeled to contain 100 mg MIC and 100.000 IU = 20.5 mg NYS.

Acetonitrile and methanol were HPLC grade (Tedia, Fairfield, OH). Ammonium acetate and glacial acetic acid were analytical grade.

HPLC conditions

The mobile phase was prepared by mixing methanol, acetonitrile and 50 mM ammonium acetate buffer (apparent pH was adjusted to 6 using glacial acetic acid) in a ratio of 60:30:10 (v/v/v). The flow rate was 1 mL/min. The injection volume was 20 μL. Quantitation based on peak area was achieved using UV detection at 230 nm. All determinations were performed at ambient temperature.

Standard solutions and calibrations

Stock standard solutions (1 mg/mL) of each drug were prepared separately in methanol for spectrophotometric methods and for HPLC. Suitable dilutions were made using the specified solvent.

CLS, PCR and PLS methods

A training set of 15 synthetic binary mixture solutions were prepared by further dilution of the stock solutions with methanol in the range of 10–90 μg/mL (MIC) and 1.8–18 μg/mL (NYS). The UV absorption spectra were recorded over the range 280–320 nm. The data points of the spectra were collected at every 0.2 nm. A validation set containing 12 synthetic binary mixtures in the ranges of 20–80 and 3.8–16.2 μg/mL1 for MIC and NYS, respectively, was prepared using the preceding stock solutions.

PLS and PCR models were applied to the UV absorption spectra of these mixtures using three latent variables for PLS and three principal components for PCR for the determination of each compound.

HPLC method

The standard solutions were prepared by further dilutions of the stock standard solutions with mobile phase to reach the concentration ranges of 4–20 μg/mL for MIC and 10–100 μg/mL for NYS. Triplicate 20-μL injections were made for each concentration and chromatographed under the specified conditions. The peak area values versus corresponding concentrations were plotted. Linear relationships were obtained.

Sample preparation

Five Monicure Plus vaginal suppositories were accurately weighed and finely powdered in a mortar. An amount of the suppository mass equivalent to one suppository content (100 mg of MIC and 20.5 mg of NYS) was dissolved in 60 mL of methanol. After 30 min of warming and mechanical shaking, the solution was filtered in a 100-mL volumetric flask. The residue was washed twice, each with 10 mL of the solvent. The volume was completed to 100 mL with methanol. Further dilutions of the filtrate were conducted with mobile phase (for HPLC method) or methanol (for spectrophotometric methods) to reach the calibration range.

Results and Discussion

Figure 1 shows the zero-order absorption spectra of MIC and NYS in methanol. It is clear that the spectra of the two drugs display considerable overlap throughout the wavelength range. Due to the presence of this spectral interference, HPLC and chemometric methods were necessary for the simultaneous determination of the two drugs in vaginal suppositories.

HPLC method

The primary target in developing this LC method is to achieve the simultaneous determination of MIC and NYS. The mobile phase composition and pH of 50 mM ammonium acetate were studied and optimized. A successful separation was obtained with a mobile phase consisting of methanol, acetonitrile and 50 mM ammonium acetate buffer (pH 6) in a ratio of 60:30:10 v/v at a flow rate of 1 mL/min.

Increasing the acetonitrile concentration to more than 40% led to inadequate separation of MIC and NYS. At lower acetonitrile concentration (<30%), separation occurred, but with excessive tailing for the MIC peak. Variation of the apparent pH of the 50 mM ammonium acetate buffer resulted in a low capacity factor (k) value for MIC at apparent pH 3.5, with loss of peak symmetry for NYS. At apparent pH 5, improved resolutions were observed for the two drugs. However, at apparent pH 6, optimum resolution with reasonable retention time was observed.

The specificity of the HPLC method is illustrated in Figure 2, in which complete separation of the two drugs was observed. The average retention time ± standard deviation (SD) for NYS and MIC were found to be 3.35 ± 0.04 and 6.67 ± 0.05 min, respectively, for 10 replicates.

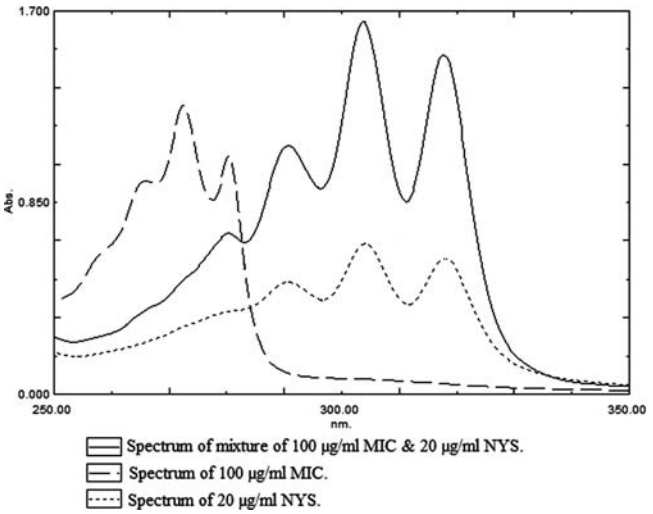

Figure 1. UV zero-order absorption spectra of 100 μg/mL miconazole (dashed line), 20 μg/mL nystatin (dotted line) and a mixture of 100 μg/mL miconazole and 20 μg/mL nystatin (straight line) in methanol.

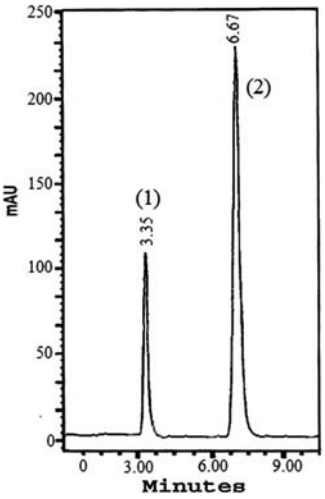

Figure 2. HPLC chromatogram of a 20-μL injection of a synthetic mixture containing 10 μg/mL nystatin (peak 1) and 4 μg/mL miconazole (peak 2) in methanol.

CLS, PCR and PLS methods

Construction of CLS, PCR and PLS models

The composition of the training set was orthogonally designed to obtain maximum information on each drug from the calibration procedure. The number of calibration mixtures in the training set was selected according to the rule of five. This rule states that using five times the number of samples as components provides enough samples to reasonably represent all possible combinations of different concentration values (16). A training set was prepared, as shown in Table I. The absorbance data matrix for this training set was obtained by recording the absorbances within the wavelength range of 280–320 nm at 0.2-nm intervals. A CLS model was constructed with non-zero intercept. To build this model, the computer was fed with the absorbance and concentration matrices for the training set. The calculations to obtain the *K* matrix were conducted. For the PCR and PLS models, the training set absorbance and concentration matrices, together with PLS-toolbox 2.1 software, were used for calculations. The cross validation method was employed to eliminate only one sample at a time, and then the remaining standard spectra were calibrated by PCR and PLS (17, 18). With the utilization of this calibration, the concentration of the remaining sample was predicted. This process was repeated until each standard had been left once.

Selection of the optimum number of factors

The optimum number of factors (latent variables) to be included in the calibration model was determined by computing the prediction error sum of squares (PRESS) for the cross-validated models, using a high number of factors (half the number of total standard + 1). The predicted concentrations of the two drugs in each sample were compared with the actual concentrations in these calibration samples and root-mean-square error of cross-validation (RMSECV) was calculated for each method. RMSECV indicates both of the precision and accuracy of predictions, and was recalculated upon addition of each new factor to the PLS and PCR models:

$$\text{RMSECV} = (\text{PRESS}/n)^{1/2} \quad (1)$$

where PRESS is the predicted residual error sum of squares and n is the number of calibration samples (19):

$$\text{PRESS} = \sum_{i=1}^{n} (C_i^{\text{Pred}} - C_i^{\text{Experimental}})2 \quad (2)$$

where C_i^{Pred} and $C_i^{\text{Experimental}}$ are predicted and true concentrations in μg/mL, respectively. Visual inspection was used for selecting the optimum number of factors. Three factors were found to be suitable for both PCR and PLS methods, as shown in Figures 3 and 4.

An independent set of validation synthetic mixtures containing MIC and NYS in the different compositions given in Table II was prepared and analyzed for validation. The mean percentage recoveries, SD and relative standard deviations (RSD) are indicated in Table II.

The standard error of prediction (SEP), mean squared error of prediction (MSEP), RMSECV, variance of prediction (S^2) and relative error of prediction (REP) are also used for validation (16, 20). The accuracy and precision of prediction are defined by MSEP and RMSECV (20):

$$\text{SEP} = [\text{PRESS}/n - 1]^{1/2} \quad (3)$$

$$\text{MSEP} = \text{PRESS}/n \quad (4)$$

$$\text{Bias} = \sum_{i=1}^{n} (C_i^{\text{Pred}} - C_i^{\text{Experimental}})/n \quad (5)$$

$$S^2 = \sum_{i=1}^{n} (C_i^{\text{Experimental}} - C_i^{\text{Pred}} - \text{bias})^2/n - 1 \quad (6)$$

$$\text{REP}(\%) = \text{RMSECV} \times (100/\hat{C}^{\text{Experimental}}) \quad (7)$$

where $C_i^{\text{Experimental}}$ is the true concentration, C_i^{Pred} is the predicted concentration, $\hat{C}^{\text{Experimental}}$ is the average concentration in the validation set and n is the total number of validation samples. The numerical values of SEP, MSEP, RMSECV and S^2 are listed in Table III. Small values of the results indicate the negligible error of prediction and high ability of prediction.

Table I
Concentrations of Different Mixtures of MIC and NYS used in the Training Set

Mixture	MIC (μg/mL)	NYS (μg/mL)
1	10	1.8
2	10	2
3	10	2.2
4	30	5.8
5	30	6
6	30	6.2
7	50	9.8
8	50	10
9	50	10.2
10	70	13.8
11	70	14
12	70	14.2
13	90	17.8
14	90	18
15	90	18.2

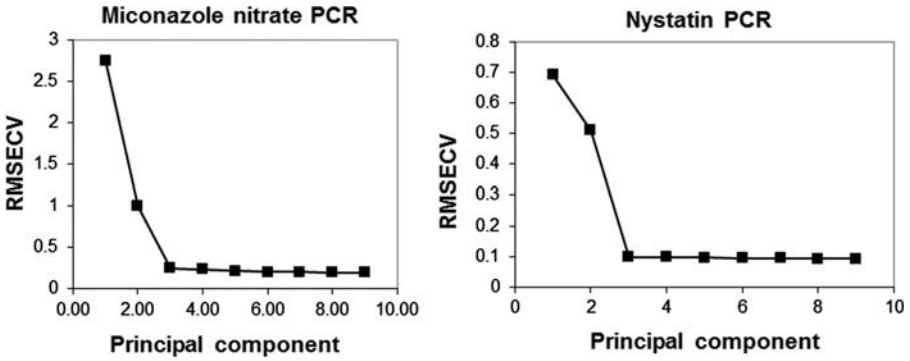

Figure 3. RMSECV plot of a calibration set prediction using cross validation (PCR model).

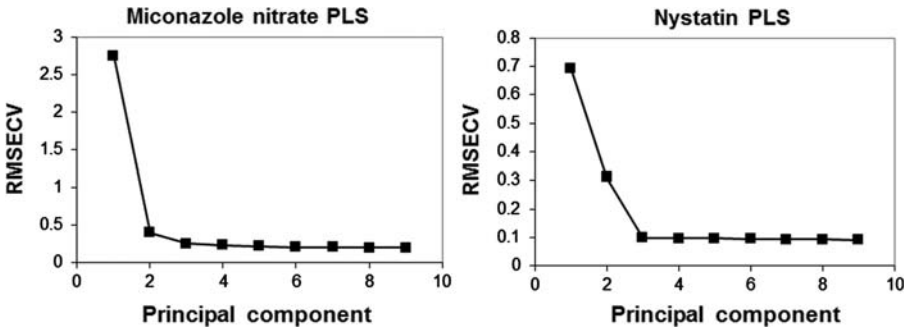

Figure 4. RMSECV plot of a calibration set prediction using cross validation (PLS model).

Table II
Assay Results of MIC and NYS Combinations in Synthetic Mixtures (Validation Mixtures) by the Proposed Chemometric Methods

Validation mixtures		Recovery (%)					
Added (μg/mL)		CLS		PCR		PLS	
MIC	NYS	MIC	NYS	MIC	NYS	MIC	NYS
20	3.8	96.72	96.31	99.45	102.85	99.45	102.85
20	4	99.74	101.86	101.44	103.01	101.44	103.01
20	4.2	101.6	99.06	99.99	99.54	99.99	99.54
40	7.8	97.66	98.73	102.11	101.79	102.11	101.79
40	8	100.38	101.54	103.36	102.33	103.36	102.33
40	8.2	104.29	103.95	97.41	101.10	97.41	101.10
60	11.8	95.95	99.49	99.83	99.37	99.83	99.37
60	12	95.49	96.91	100.93	100.17	100.93	100.17
60	12.2	97.51	97.97	96.33	99.66	96.33	99.66
80	15.8	96.47	99.39	98.57	97.77	98.57	97.77
80	16	96.97	98.95	98.57	98.89	98.57	98.89
80	16.2	98.88	98.45	99.22	98.12	99.22	98.12
Mean		98.47	99.38	99.76	100.38	99.76	100.38
± SD		2.61	2.14	1.98	1.80	1.98	1.80
RSD		2.65	2.16	1.98	1.79	1.98	1.79

Table III
Statistical Parameters of the Validation of Synthetic Mixtures using the Proposed Chemometric Methods

Parameters	MIC			NYS		
	CLS	PCR	PLS	CLS	PCR	PLS
Intercept	−0.99	−1.08	−1.08	−0.09	−0.28	−0.28
Slope	1.04	1.03	1.03	1.02	1.03	1.03
r	0.998	0.998	0.998	0.999	0.999	0.999
SE of intercept	0.81	0.72	0.72	0.13	0.08	0.08
SE of slope	0.01	0.01	0.01	0.01	0.01	0.01
Lower CL of intercept*	−2.79	−2.69	−2.69	−0.38	−0.46	−0.46
Upper CL of intercept*	0.82	0.53	0.53	0.18	−0.1	−0.1
Lower CL of slope*	1	1	1	0.99	1.01	1.01
Upper CL of slope*	1.07	1.06	1.06	1.04	1.05	1.05
LOD (μg/mL)	3.58	3.21	3.21	0.57	0.35	0.35
LOQ (μg/mL)	10.85	9.74	9.74	1.73	1.07	1.07
SEP	1.77	1.19	1.19	0.2	0.18	0.18
MSEP	2.87	1.31	1.31	0.04	0.03	0.03
RMSECV	1.69	1.15	1.15	0.19	0.17	0.17
S^2	2	1.32	1.32	0.035	0.031	0.031
REP (%)	3.39	2.29	2.29	1.95	1.69	1.69

*Confidence limit; calculated at 95% confidence limit.

Plotting of the predicted versus true concentrations is also used for validation. A straight line is expected to be obtained (20). The regression analysis for these linear relationships was conducted and the results are shown in Table III. The absence of bias was proved by determining the confidence limits for the intercept and the slope at the 95% significance level (21). Limit of quantification (LOQ) and limit of detection (LOD) are also given in Table III.

Validation of the methods

Linearity

The linearity of the HPLC method for determination of MIC and NYS was evaluated by analyzing a series of different concentrations of each drug. In this study, six concentrations were chosen, ranging between 4–20 μg/ml for MIC and from 10–100 μg/mL for NYS. Each concentration was repeated

three times, to provide information on the variation in peak area values between samples of the same concentration. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p = 0.05$) different from zero (Table IV). Characteristic parameters for regression equations of the HPLC method obtained by least-squares treatment of the results are given in Table IV.

*LOQ and LOD*The LOD and LOQ are given in Table IV. The SD of the response and the slope was used for calculating the detection and quantitation limits (22) as follows: LOD = 3SD/Slope and LOQ = 10SD/Slope.

Precision

The intra-day and inter-day variations of the method were determined using three replicate injections of three different concentrations, which were prepared and analyzed on the same day and on three different days over a period of two weeks, respectively (Table V). These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs.

Accuracy

The interference of excipients in the pharmaceutical formulations was studied in detail by the CLS, PCR, PLS and HPLC methods. For this reason, the standard addition method was applied to the pharmaceutical formulation containing these compounds. In application of standard addition method to the pharmaceutical formulation, the mean percentage recoveries and their standard deviations for the proposed methods were calculated (Table VI). According to the obtained results, satisfactory precision and accuracy were observed for these methods. Consequently, the excipients in pharmaceutical formulation do not interfere in the analysis of these compounds in the pharmaceutical formulation.

System suitability

Resolution (R_s) is a measure of the degree of separation between adjacent peaks. A value of 1.5 for resolution implies a complete separation of the two compounds (23). Additionally, British Pharmacopoeia specifies that the symmetry factor of a principal peak must be between 0.8 and 1.5 (23). Resolutions and other system suitability parameters were calculated for MIC and NYS. Their values were found to be acceptable (Table VII).

Ruggedness and robustness tests

As recommended in the ICH guidelines and the Dutch Pharmacists guidelines, a robustness assessment was performed during the development of the analytical procedure (24). The ruggedness (25) of the method was assessed by comparison of the intra-day and inter-day assay results for MIC and NYS that were performed by two analysts. The RSD values for intra-day and inter-day assays of MIC and NYS in the Monicure Plus vaginal suppositories performed in the same laboratory by two analysts did not exceed 3.8%, indicating the ruggedness of the method. In addition, the robustness of the method was investigated under a variety of conditions, including changes of the flow rate, PH and mobile phase composition (26).

Table IV
Calibration Curve Data for MIC and NYS using the HPLC Method

Regression parameters	MIC	NYS
Regression coefficient (*r*)*	0.9997	0.9993
Calibration range (μg/mL)	4–20	10–100
LOD (μg/mL)	0.36	3.4
LOQ (μg/ml)	1.1	10
Slope ± SD	3.47 ± 0.06	0.5 ± 0.01
CL of the slope†	−0.45–1.71	0.49–0.53
Intercept ± SD	0.62 ± 0.95	0.96 ± 1.01
CL of the intercept†	3.4–3.55	−0.36–2.11
Number of points (*n*)	6	6

*Degrees of freedom: 5.
†Calculated at 95% confidence limit.

Table V
Intra-Day and Inter-Day Precision of MIC and NYS Standard Solutions by the HPLC Method

Compound	Theoretical concentrations (μg/mL)	Inter-day measured concentrations (μg/mL)		Inter-day measured concentrations (μg/mL)	
		Mean	RSD%	Mean	RSD%
	4	3.98	0.06	4.51	0.33
MIC	12	12.34	0.22	11.89	0.21
	20	19.75	0.14	20.45	0.13
	20	19.88	0.27	19.98	0.26
NYS	60	59.69	0.52	59.66	0.62
	100	100.33	0.23	99.81	0.23

Table VI
Determination of MIC and NYS in Monicure Plus Vaginal Suppositories using the Proposed Chemometric and HPLC Methods*

	CLS	PCR	PLS	HPLC
MIC				
Mean recovery (%) ± SD	100.14 ± 0.55	100.12 ± 0.55	100.12 ± 0.55	99.98 ± 0.34
t	1.32	1.32	1.32	(2.31)†
f	2.88	2.88	2.88	(6.39)†
NYS				
Mean recovery (%) ± SD	100.45 ± 0.78	100.39 ± 0.78	100.39 ± 0.78	100.12 ± 0.23
t	0.88	0.88	0.88	(2.31)†
f	1.22	1.22	1.22	(6.39)†
Standard addition technique‡				
Mean recovery (%) ± SD for MIC	100.21 ± 0.66	100.21 ± 0.66	100.21 ± 0.66	100.15 ± 0.6
Mean recovery (%) ± SD for NYS	100.13 ± 0.57	100.13 ± 0.57	100.13 ± 0.57	100.17 ± 0.51

*Note: Monicure Plus vaginal suppositories labeled to contain 100 mg MIC and 20.6 mg NYS per suppository.
†Theoretical values for *t* and *F* at $P = 0.05$.
‡For standard addition of 50% of the nominal content.

Table VII
Parameters Required for System Suitability Testing of the Proposed HPLC Method

Parameters	MIC	NYS
Resolution (R_s)	1.7	
Selectivity (α)	2.8	
Symmetry factor (*T*)	1.01	1.04
Capacity factor (*k'*)	3.44	1.23
Number of theoretical plates (*N*)	2,743	1,557
HETP (cm/plate)	0.01	0.02

Selectivity

Method selectivity was achieved by preparing eight laboratory-prepared mixtures of the studied compounds at various concentrations within the linearity range. The laboratory-prepared mixtures were analyzed according to the procedures described under the proposed methods. Satisfactory results were obtained (Table VI), indicating the high selectivity of the proposed methods for simultaneous determination of MIC and NYS.

Analysis of MIC and NYS in Monicure Plus vaginal suppositories

The four methods were applied to the determination of MIC and NYS in commercial Monicure Plus vaginal suppositories. Recoveries were calculated using external regression equations. No interfering peaks were observed from any of the excipients. The assay results revealed satisfactory accuracy and precision, as indicated by the recovery and SD values (Table VI).

Recovery data resulting from the proposed CLS, PCR, PLS and HPLC methods were statistically compared with those of the reported HPLC methods: the method of Akay *et al.* (9) for MIC and that of Groll *et al.* (12) for NYS, using one way ANOVA. It was found that the calculated *F* values did not exceed the critical value of the *F*-ratio at $\alpha = 0.05$, indicating no significant differences between the proposed and reported methods (Table VIII)

Conclusion

Chemometric and HPLC methods have been developed for the simultaneous determination of miconazole and nystatin in their synthetic binary mixtures and vaginal suppositories. The results obtained using chemometric methods (CLS, PCR and PLS) were compared to those of the proposed HPLC method and no significant difference was observed between the methods. The HPLC method is superior with regard to identification and specificity. However, the chemometric methods are less expensive and do not require sophisticated instrumentation prior separation steps. The presented methods are suggested to be used in the routine analysis of miconazole and nystatin in their synthetic binary mixtures and vaginal suppositories.

Table VIII

ANOVA Test for Statistical Comparison of the Recovery Data Results Obtained by the Proposed Chemometric and HPLC Methods and Reported Methods*

Compound	Source of variation	Sum of squares	Degree of freedom	Mean sum of squares	*F*-ratio†	*P*-value
MIC	Between groups	2.78	4	0.69		
	Within groups	13.62	20	0.68	1.02	0.42
	Total	16.4	24			
NYS	Between groups	3.21	4	0.8		
	Within groups	18.06	20	0.91	0.89	0.49
	Total	21.27	24			

*Note: Reported methods are from Akay *et al.* (9) for MIC and Groll *et al.* (12) for NYS.
†The critical value of the *F*-ratio is 2.87 at $\alpha = 0.05$.

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