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SPECTROPHOTOMETRIC METHOD FOR QUANTITATIVE DETERMINATION OF NYSTATIN ANTIFUNGAL AGENT IN PHARMACEUTICAL FORMULATIONS

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The low solubility of Nystatin causes biopharmaceutical problems decreasing the bioavailability or biological availability, compromising the bioanalytical analysis. The aim of this study is to achieve a quantitative analysis method with appropriate sensitivity and suitable robustness for this drug substance. The controlled photochemical transformation of Nystatin solution was conducted with a LUP 6W lamp. The maximum slope (in absolute value) of the curve associated with 322 nm radiation is recorded at the beginning of the irradiation; based on this, it can be established the optimal irradiation time (30 minutes) when analysis were carryied out. The average value of the determinations is not far from the expected value. The method for quantification of Nystatin in pharmaceutical formulations, based on the photosensitivity and selective photo-transformation of the active substance, has proved to be reliable for the analytical control of these types of pharmaceutical formulations.

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Keywords: Spectroscopy, Nystatin, pharmaceutical formulations

1. Introduction

Nystatin antifungal agent was isolated in 1950 from *Streptomyces noursei* and the molecular structure was completely elucidated in 1976 by nuclear magnetic resonance spectroscopy (1H RMN) and mass spectrometry (MS) [1, 2].

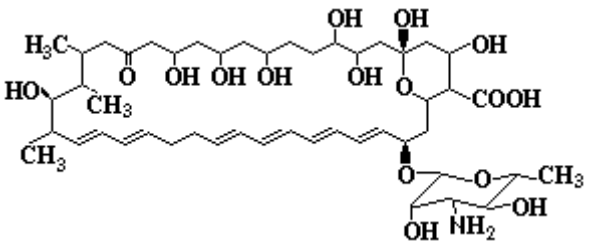

Fig. 1. Molecular structure of Nystatin

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From structural point of view, Nystatin is a macrocyclic molecule containing a macrocycle formed of 38 atoms (37 carbon atoms and one oxygen atom), 6 C = C double bonds, 10 hydroxyl groups, 4 ether bonds (–O–), a carboxyl group and a primary amine group. It has a CAS Registry Number 1400–61–9, molecular formula of $C_{47}H_{75}NO_{17}$ and a molecular weight of 926.13 g/mol. Systematic name by the International Union of Pure and Applied Chemistry (IUPAC) is 20–(4–amino–3,5–dihydroxy–6–methyl–oxan–2–yl), oxy–4,22,24,28,29,32,34,36–octahydroxy–2,3,5–trimethyl–26,38–dioxo–1–oxacyclooctatriaconta–6,8,12, 14,16,18–hexaene–23–carboxylic acid (Figure 1) [3]. The quantitative analysis of the products based on Nystatin is hampered by the limited solubility of this compound [4], which is practically insoluble in water [5], very slowly soluble in alcohol and chloroform and generally in the usual solvents [6]. Nystatin presents acceptable solubility in aqueous alkaline medium, in dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) [7]. These solvents limit seriously the applicability of the analytical methods [8]. The low solubility compromises the customary analysis methods [9]. For this reason, quantitative determinations based on physico-chemical methods seem to be adequate presenting satisfactory accuracy, precision, selectivity, sensitivity, robustness, and advantageous traceability [10]. From this point of view, the absorption spectrophotometry in ultraviolet range seems to correspond to the demands: it is a relatively simple, rapid and especially sensitive method [11]. Unfortunately, the main disadvantage of the solvents that somewhat dissolve the compound (dimethyl sulfoxide, dimethyl formamide) consists in the rather extensive spectral region with important optical absorption [12], obscuring the absorption bands of the compound of interest [13], especially at short wavelengths [14]. Another disadvantage is the usual presentation form of pharmaceutical formulations for which the analytical determination is achieved [15]. Pharmaceutical formulations usually contain some ingredients (glycerine, vitamin A, anesthesine, etc.), with significant ultraviolet absorption as well [16, 17].

2. Materials and methods

Reagents and solutions. All reagents usedwere of analytical grade (Merck). When necessary, appropriate dilutions were made to the desired concentrations during the sample preparation. The solvents used were of chromatographic purity. The reference substance - Nystatin - was purchased fromFluka. Dioxane,usedassolvent, was of spectroscopicpurity (Merck). The solution with Nystatin, subject to analysis, hadthe following composition:

Substance	***Quantity***
Nystatin	1.5 g
Glycerol	100 g
Vitamin A	½ bottle
Sodium bicarbonate	5 g
Water	10 g
Anesthesine (benzocaine)	1.5 g
Diluted alcohol	20 mL
Total	**139 g**

The controlled photochemical transformation of the standard solutions and the sample solution was performed with a LUP 6W lamp with 6 W output power in the spectral range of 350–400 nm ("Luxten Lighting").

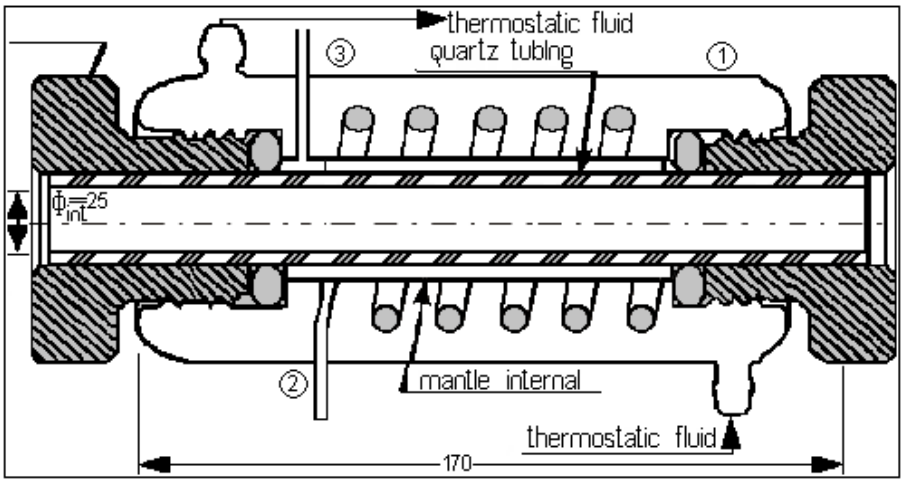

Fig. 2. Scheme of the photochemicalreactor used for controlled irradiation of the solutions (dimensions are giveninmm).

Figure 2 shows the scheme of the photochemical reactor used for controlled irradiation of standard solutions and samples. The ultraviolet radiation source (not represented in Figure 2) is inserted in the centrum of tubular reactor. The interior of the reactor is kept at constant temperature (30°C) by circulating a fluid through the outer mantle (1). Solutions to be irradiated are introduced through the tube (2) and during transition through helical pipeline they reach the temperature of the circulating fluid. Finally, solutions enter the inside mantle whose inner wall is manufactured of quartz glass (transparent in the ultraviolet spectral range). The distance between the light source and solutions in the inner mantle is 5 mm [18].

Preparation of standard solutions. Figure 3 shows the preparation steps of standard solutions. Based on the recipe of the solution preparation, expected content of Nystatin is 1.07% (w/w). The preparation stages of standard solutions and preliminary processing of samples take into account this expected value. 10.0 mg Nystatin reference substance is weighed at semi micro analytical balance (it features a 0.01 mg weighing resolution) and introduced in a graduated flask with 50 mL nominal volume. A quantity of dioxane–water mixture (7:3) is added and after complete dissolution of mixture material, volume is completed to mark with same solvent. A stock solution is obtained with 200 mg/L Nystatin concentration. 25 mL of this solution is diluted to 100 mL with dioxane–water solvent (7:3).

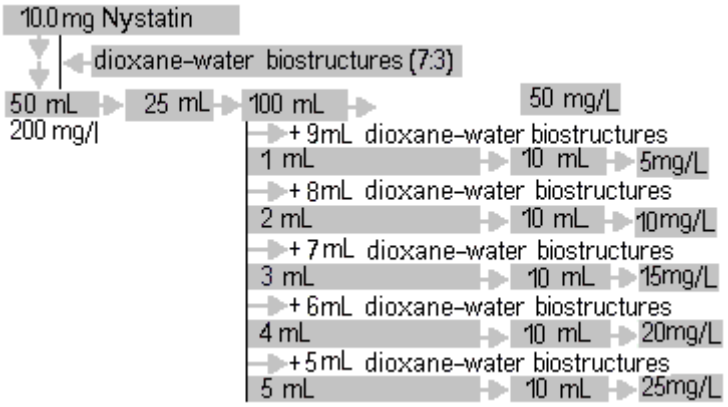

Fig. 3. Steps of standard solutions preparation

From the obtained solution a series of standard solutions, each of 10 mL, is prepared with the concentrations of 5, 10, 15, 20 and 25 mg/L. The absorption spectra of standard solutions are registered in spectral range 240–400 nm using quartz cuvettes with optical path of 5 mm.

Performance analysis. Figure 4 shows the operation sequence for dilution of the real samples (solution with Nystatin). 1 mL of the homogenized original sample (solution) is pipetted into a volumetric flask with the nominal volume of 50 mL.

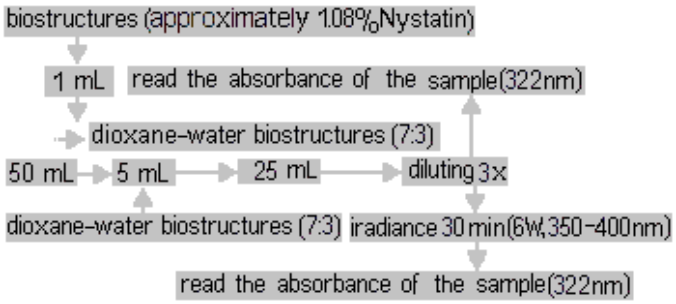

Fig. 4. Dilution sequence of the primary samples for spectrophotometric analysis.

Dioxane–water solvent mixture (7:3) is added to flask up to mark and is well shake to homogenisation. Before the segregation of the undissolved phase, 5 mL of the suspension is transferred in another flask (25 mL) and same dioxane–water solvent is added to full volume. This time, a homogeneous transparent solution is formed. The prepared solution is diluted three times (10 mL and 20 mL solvent) and the obtained solution is divided into two parts. The absorbance of first portion is determined at 322 nm in cuvette with thickness of 5 mm. The second portion is subjected to irradiation using a lamp emitting in ultraviolet (350–400 nm) for 30 minutes, after which is determined the optical absorbance under identical conditions with those applied to the first portion. The difference between the absorbance values is used as the analytical signal, proportional to the Nystatin concentration.

Validation. After the initial establishment of the experimental conditions, the pharmacopoeia validation requirements were followed using the solution suitability, linearity, precision (repeatability and intermediate precision), accuracy and robustness of the assay.

Data Evaluation and Statistical Analysis. Statistical analysis of results was limited to arithmetic mean and relative standard deviation (RSD).

3. Results and discussion

Figure 5 shows the absorption spectrum of pure Nystatin in dioxane–water solution (volume ratio 7:3).

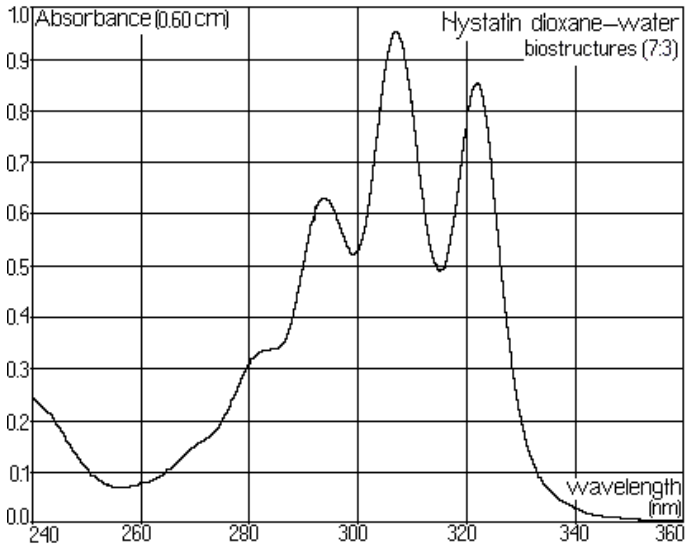

Fig. 5. Absorption spectrum of Nystatin solution in dioxane–water mixture (7:3)

Three well–defined optical absorption maximums are observed. In principle, each of three maximum could form a basis for quantitative determination. In real samples, it is not possible a simple performance of the analysis, because of significant amount of excipients, whose absorptions overlap with the signal of pure Nystatin [19]. It can be used the fact that the interest compound presents photosensitivity, and after irradiation the interest compound modifies its optical absorbance, unlike expected ingredients [20]. This change is used as analytical signal. Figure 6 shows the superposed absorption spectra of the initial Nystatin solution and after irradiation for 30 and 60 minutes as well.

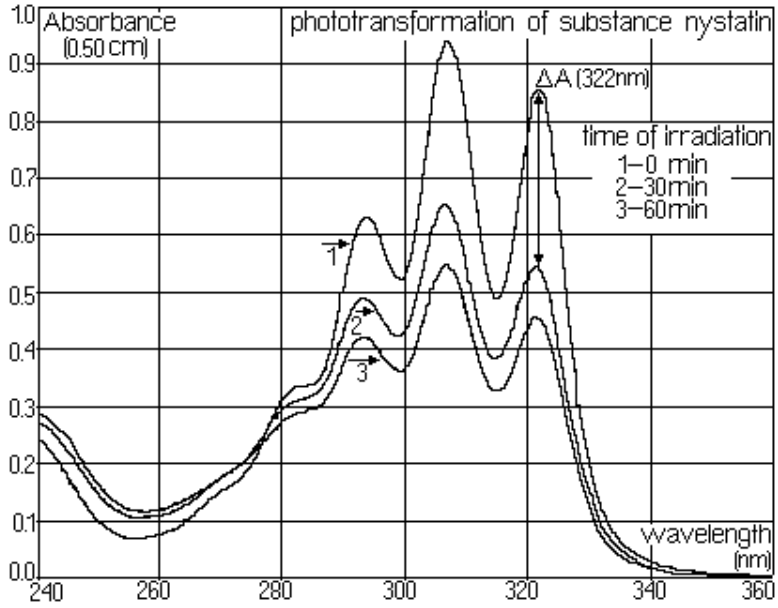

Fig. 6. The effect of irradiation on the absorption spectrum of Nystatin compound.

It can be observed that all the absorption maximums of the compound are affected by irradiation (decreasing with time of irradiation), but the effect is most pronounced at 322 nm.

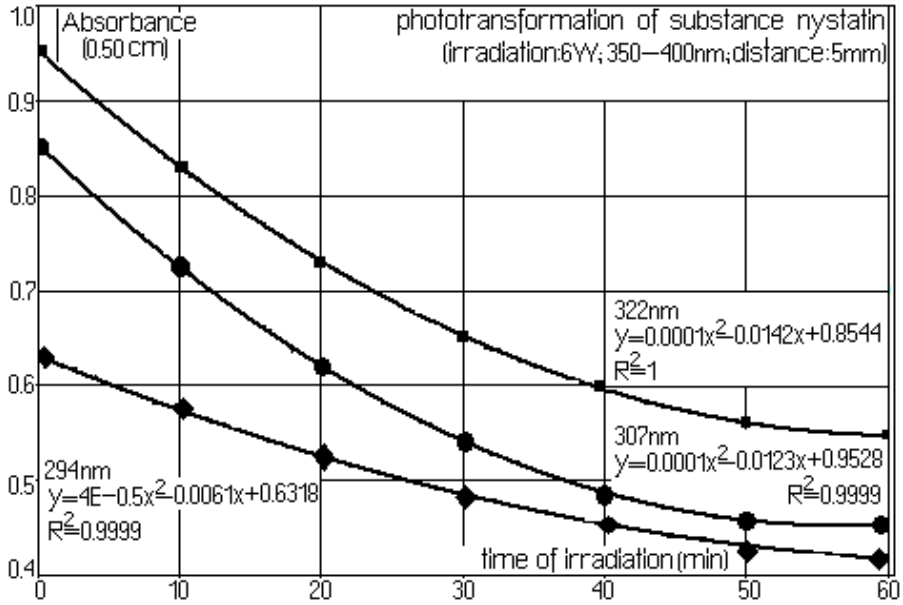

Fig. 7. The variation of absorbances by the irradiation time

The analytical measurements can be performed at this wavelength. The choice of the wavelength at 322 nm (instead of some smaller wavelengths) presents the advantage that the major ingredients (without chromophore) can be neglected at this wavelength. It is noted that the absorbance values decrease with the increasing of the irradiation dose. Figure 7 shows the absorbance variations at the three maximum vs. the irradiation time. The fastest decrease of the radiation induced absorbance is observed at 322 nm. It is logical to accept as analytical signal the absorbance variation produced by the irradiation at this wavelength. It is noted that the maximum slope (in absolute value) of the curve associated with 322 nm is recorded at the beginning of the irradiation, and the required irradiation time for analyses is 30 minutes. It is interesting that the figurative points in the graph in Figure 7 match, almost perfect, with a second order polynomial relationship. It is required to test linear dependence of absorbance change at 322 nm on Nystatin concentration for a constant irradiation time, respecting strictly identical conditions of irradiation.

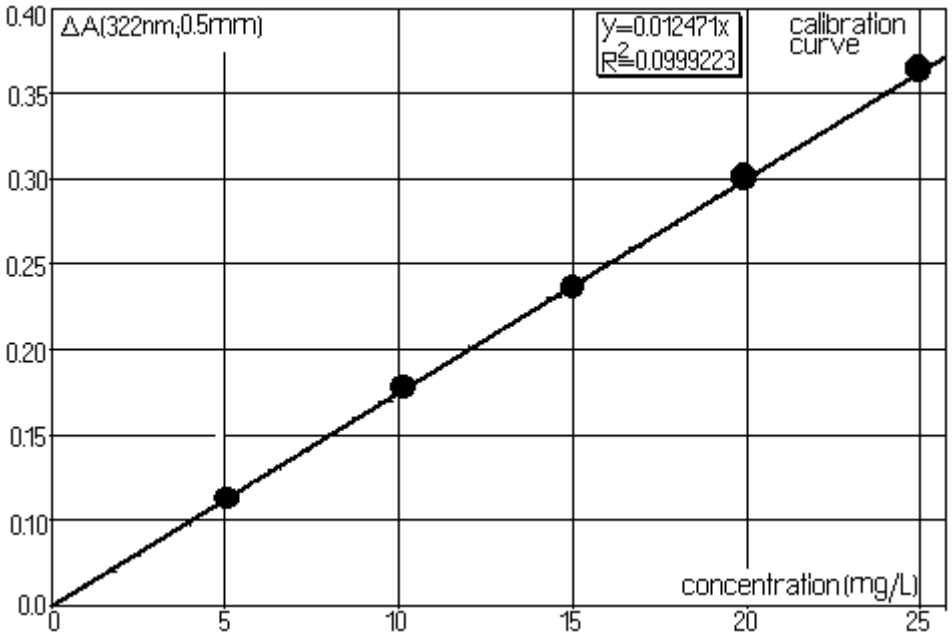

Fig. 8. Calibration line ΔA (322nm, 5 mm) vs. concentration (mg/L).

Table I presents the absorbance changes at 322 nm (□A) of the standard solutions, prepared as shown in Figure 3, for 30 minutes irradiation time.

Tabel 1. The absorbance variation at 322 nm (ΔA) of the standard solutions.

Concentration (mg/L)	**ΔA (322 nm; 0.5 cm; irradiation 30 min.)**
5	0.0625
10	0.1288
15	0.1840
20	0.2480
25	0.3131

Figure 8 shows the linear correlation of the data included in Table 1. It is noticed that the correlation between □A values (322 nm, 0.5 cm) and Nystatin concentrations (mg/mL) is linear and can form a basis for a quantitative determination. The prior processing of the Nystatin solution sample is made according to the dilution scheme shown in Figure 4. Figure 9 shows the two superposed spectra of a solution derived from the sample solution, before and after irradiation for 30 minutes.

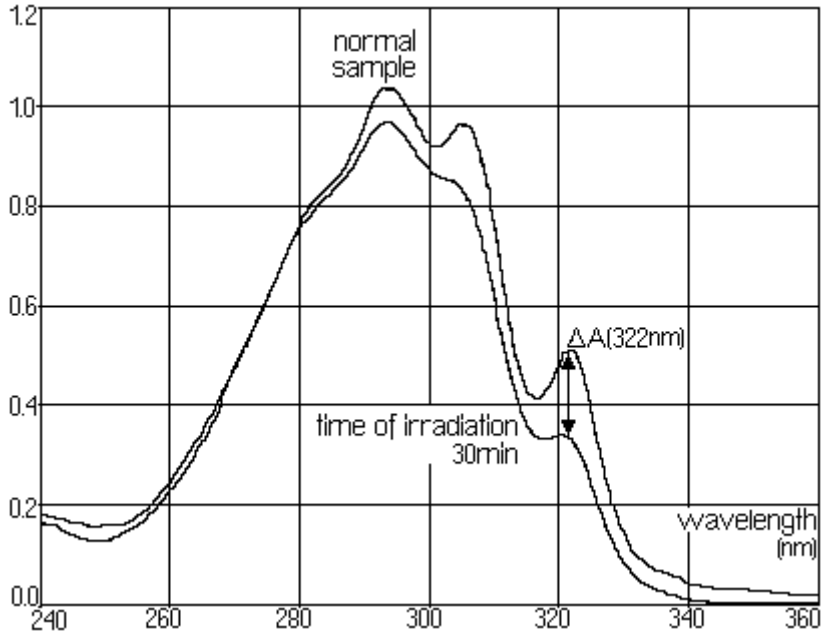

Fig. 9. Superposed spectra of a solution derived from Nystatin solution sample before and after irradiation for 30 minutes.

Table 2 presents data obtained for 5 samples of Nystatin solution, provided by the same producer.

Tabel 2. Values obtained for Nystatin sample solutions.

ΔA (322 nm; 0.5cm; 30 min.)	*Concentration (mg/mL)*	*Nystatin content in solution (%)*
0.1729	13.864	1.039
0.1741	13.960	1.047
0.1733	13.896	1.042
0.1752	14.048	1.053
0.1758	14.097	1.057
	Average	**1.048**
	Std. Dev.	**0.0075**

The average Nystatin content resulting from the five determinations differs from the expected value not more than 3.15 % of the average. Standard deviation (RSD) of the individual values around the average is 0.716 % of the average. Statistical analysis was limited to these two statistic parameters.

4. Conclusions

The pharmaceutical mixture that contains Nystatin presents photosensitivity and unlike the ingredients envisaged the irradiation changes its optical absorbance. Absorption spectrophotometry in the ultraviolet domain appears to correspond to the purpose of this research being a relatively simple, fast and sensitive quantification method of the solution than contains Nystatin.

In conclusion, the analytical method based on exploiting photosensitivity and selective phototransformation of the active substance proved to be reliable for analytical control of these types of mixtures.

Acknowledgments

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