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Development and Validation of Analytical Methods for the Identification in Canprophem - AK® Suppositories with the New Original Polyene Antibiotic

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ABSTRACT

Introduction: The paper presents analytical methods for the identification of Roseofungin active pharmaceutical ingredient (API) using thin layer chromatography (TLC) and spectrophotometry in the ultraviolet region, for the drug CANPROFEM - AK® containing the original polyene antibiotic Roseofungin in the form of suppositories. The study established optimal conditions for the experiment, including sample preparation; the Specificity validation characteristic was examined for each method.

Results: Method 1 employed the TLC plate pre-coated with a layer of F254 silica gel using n-butanol saturated with purified water as a mobile phase; the substance was identified in UV light at a wavelength of 365 nm. The method makes it possible to uniquely and specifically identify roseofungin in the presence of placebo components.

In method 2, the maximum of the ultraviolet absorption spectrum of the solution being tested coincides with the maximum of the ultraviolet absorption spectrum of the reference sample solution (364±2 nm).

Conclusion: The methods are suitable for solving the intended objectives and are included in the draft analytical normative document on the finished product quality control by the Identification characteristic.

Keywords: roseofungin, CANPROFEM-AK® suppositories, thin-layer chromatography, absorption spectrophotometry in the ultraviolet and visible regions, validation, identification.

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INTRODUCTION

Vulvovaginal candidiasis is an infectious disease of the external genitalia and vaginal mucosa caused by *Candida* fungi [1-3]. The genus *Candida*, from a medical point of view, belongs to one of the largest genera of yeast-like microorganisms and includes about 200 species [4]. At the same time, about 30 species of *Candida* belong to conditional pathogens [5]. However, only 7 of them cause more than 90% of invasive infections, including *C. albicans* (58.6% to 95%), *C. crusei* (17.2%), *C. dubliniensis* (9.2%), *C. glabrata* (3.4%), *C. tropicalis* (2.3%), *C. parapsilosis* (2.3), *C. guilliermondii* (2.3%) [6]. Approximately 75% of adult women have at least one episode of vulvovaginal candidiasis in their life, and about half of these women experience more than one relapse; 5-8% have several episodes each year [7–9]. It should be noted that vulvovaginal candidiasis is not attributed to sexually transmitted infections, a decrease in immunity is the major factor in the appearance of candidiasis. Vulvovaginal candidiasis is quite often observed in pregnant women, women taking contraceptives with high estrogen content, antibiotics, immunosuppressants, having a history of diabetes, chronic anemia, allergies, cancer, AIDS and other diseases that reduce the immune response [1, 2, 6, 9, 10]. Candidiasis is mainly treated with antimycotic drugs (polyene antibiotics, imidazole derivatives, triazole, pyridones, etc., probiotics) [9, 11-13] both orally and vaginally [14], using various dosage forms (suppositories/pessaries, tablets, capsules, solutions, syrups, ointments, creams, syringing, etc.) [14-17]. Given the global problem of antibiotic resistance [18, 19], the development and production of new drugs for the treatment of this pathology is quite relevant and timely trend [17, 19, 20].

SPC for Microbiology and Virology LLP (Almaty, Republic of Kazakhstan) together with the School of Pharmacy under S.D. Asfendiyarov National Medical University JSC (Almaty, Republic of Kazakhstan) carries out pharmaceutical development of a new drug in the form of suppositories with the original substance [22, 23], which is a polyene antibiotic Roseofungin (at a concentration of 2%), produced by *Streptomyces roseoflavus v. roseofungini AS-20.14* (the structure is shown in Figure 1) [24, 25]. The substance has the proven high activity against pathogens of mycotic infections, including the most dangerous causative agents of superficial and deep mycoses (Table 1), and possesses a pronounced antiviral (virucidal) activity [24-26].

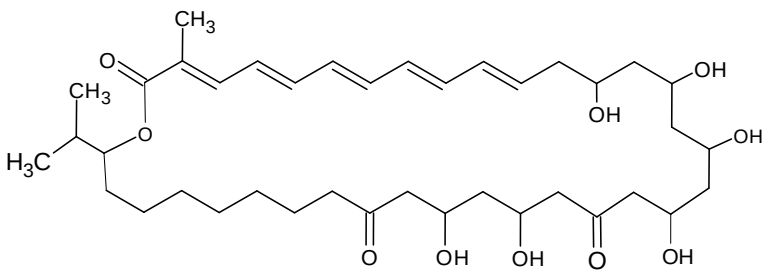

Figure 1: Structural formula of polyene antibiotic Roseofungin

Table 1: Spectrum of antifungal activity of polyene antibiotic Roseofungin [25]

Test organism	Minimum inhibitory concentration, mg/ml
Trichophyton gypseum	0.53
Tr. crateriforme	0.78
Tr. violaceum	1.11
Epidermophyton rubrum	1.11
Ep. K.-W.	0.60
Achorion schonleini	0.60
Microsporum lanosum	0.78
Cryptococcus neoformans	4.69
Sporotrichum schenckii	7.29
Hormodendrum sp.	5.21
Aspergillus fumigatus	8.33
Emmonsia crescens	6.25
Candida albicans	1.33
C. guilliermondii	1.11
C. krusei	1.11
C. tropicalis	1.61
C. utilis	1.65
C. pseudotropicalis	1.61
C. gellaris	1.33
Botrytis cinerea	0.78
B. allii	1.11
Fusarium vasinfectum	4.01
F. solani	1.33
F. oxysporum var. solani	4.01
F.oxysporum f. melonis	1.61
F. bulbigenum var. blasticola	3.90
Rhizoctonia aderholdii	1.11
Rh. solani	0.78
Helminthosporium sativum	1.11
Alternaria humicola	1.33
Sclerotinia libertiana	1.11
Verticillium dahliae	0.78
Phomopsis	0.78
Aspergillus niger	4.60
Penicillium granulatum	1.33
Trichoderma lignorum	4.60
Tr. koningi	4.60
Tr. album	4.60
Tr. glaucum	4.60

Pharmaceutical development of a new drug provides in particular for the development of quality control methods for raw materials, intermediate products, and finished drug during its production [26].

To identify polyene antibiotics (nystatin, amphotericin, natomecin, levorin, etc.), chromatographic (thin layer and liquid) [27-29] and spectral (in infrared, ultraviolet, and visible regions) [30-35] methods as well as qualitative reactions [31-35] are most frequently used. In this case, preference is given to the use of physicochemical methods [30, 33, 34, 35], which make it possible not only to specifically identify the active molecule of the active substance due to personalized spectral characteristics and chromatographic behavior, but also to separate the active pharmaceutical ingredient (API) from possible concomitant substances (impurities, placebo components, etc.).

An analytical normative document (AND) on the identification of Roseofungin API recommends two methods using thin layer chromatography (TLC) and absorption spectrophotometry in the ultraviolet region. It is advisable to study the applicability and evaluate the suitability of these methods for the identification of the active substance in the suppository dosage form.

After evaluating the suitability, the proven methods should pass through a validation phase in accordance with the requirements of the State Pharmacopoeia of the Republic of Kazakhstan [30] and ICH guidelines [36].

The purpose of the study was to evaluate the suitability and validate methods for the identification of roseofungin in the new original drug CANPROFEM - AK® by TLC and absorption spectrophotometry in the ultraviolet region.

MATERIALS AND METHODS

Suppositories with Roseofungin API produced at the manufacturing site of the SPC for Microbiology and Virology LLP were used as the objects of study; suppository base consisting of Witepsol W-35 and Roseofungin AS Enterprise Reference Standard (ERS) served as a placebo.

Used equipment included the following: Pioneer TM electronic scales (Ohaus, USA); HF-400 refrigerator (Pozis, Russia); TSO-200 SPU thermostat (Smolensk SKTB SPU OJSC, Russia); UFS-254-365 chromatographic irradiator (Russia); 1002-1013 Wasserbader water bath (GFL, Germany); Kieselgel-60 chromatography plates with a layer of F254 silica gel (0.20 mm sorbent layer thickness, 60 μm particle size) (Merk, Germany); glass chromatography tank (25×25×9 cm); UV mini-1240 spectrophotometer (Shimadzu, Japan). All chemicals used were of reagent grade (RG) and analytical reagent grade (ARG). The devices in use (measuring instruments) were qualified.

To evaluate the suitability of API identification methods with regard to suppositories, it was first necessary to assess the influence of the suppository base (placebo) on the chromatography process and the additional contribution of placebo to the optical density and to select the optimal weighed portion of the drug under study.

Validation testing of analytical methods for compliance with the Specificity characteristic was carried out by studying the suppositories CANPROFEM - AK®, Roseofungin ERS, and suppository base (placebo). These characteristics were evaluated using 9 determinations, three samples were tested in triplicate.

Method 1

The determination is carried out by TLC in accordance with the requirements of [30, 2.2.27].

Mobile phase: *n-butanol P saturated with purified water P*

Test solution

0.250 g of the drug is placed in a 10.0 ml volumetric flask, 5 ml of 96% *ethanol P* are added; the mixture is heated in a water bath at a temperature not exceeding 45 ° C until the suppository mass is completely melted, then cooled, and the resulting solution is adjusted to the mark with the same solvent, stirred and filtered.

Reference solution

5.0 mg of *Roseofungin ERS* is placed in a 10.0 ml volumetric flask, dissolved in 5 ml of 96% *ethanol P*, cooled, and the final solution is adjusted to the mark with the same solvent and mixed.

All solutions used should be freshly prepared.

10 μl of the test solution (5 μg of API) and the reference solution (5 μg of ERS) are applied on the start line of the chromatographic plate. The plate is dried in a stream of air for 5-10 minutes, placed in the chamber containing a mobile phase, and chromatographed in an ascending manner. When the mobile phase front passes 80-90% of the plate length from the start line, the plate is taken out of the chamber, dried in air for 30 minutes and detected in UV light at a wavelength of 365 nm.

One adsorption zone should be detected on the chromatogram of the test solution, which fluoresces yellow at the level of the adsorption zone of roseofungin on the chromatogram of the reference solution, corresponding to it in size and shape.

Method 2

The determination is carried out by absorption spectrophotometry in the ultraviolet region [30, 2.2.25].

Test solution

50.0 mg of the drug is placed in a 100 ml conical graduated flask, 20 ml of 96% *ethanol P* are added; the mixture is melted when heated at a temperature of 45° C in a water bath under constant stirring, cooled, and the final solution is adjusted with 96% *ethanol P* to the 100 ml mark, stirred and filtered.

The freshly prepared solution should be used.

Reference solution

25.0 mg of roseofungin (ERS) is placed in a 25 ml volumetric flask, dissolved in 96 ml of 96% *ethanol P*; the final solution is adjusted to the mark with the same solvent. 1.0 ml of the resulting solution is transferred to a 100 ml volumetric flask, and the volume of the solution is adjusted with 96% *ethanol P* to the mark.

The optical density of the test solution is measured using a spectrophotometer in the 200-600 nm range in a cuvette with a layer thickness of 10 mm, using 96% *ethanol P* as a compensation solution. The optical density of the reference solution is measured in parallel.

The UV absorption spectrum of the test solution should have a maximum at a wavelength of 364±2 nm, which corresponds to the maximum absorption of roseofungin in the reference solution.

RESULTS AND DISCUSSION.

Method 1:

TLC suitability testing

The results of selection of the optimal weighed portion of the drug are presented in Table 2 and Figure 2. The data in the Table show that an increase in the suppository mass in the weighed portion affects R_f of roseofungin, reducing it by more than 15% compared to Rf of RS (0.65±0.01 versus 0.55±0.01 with 500 mg portion of the drug). It was shown that a decrease in the weighed portion and, as a consequence, of the suppository mass in it, led to the levelling of the base negative effect on the Rf value of roseofungin.

Table 2: Effect of the drug weighed portion on the value of retardation factor of roseofungin in chromatography

	Roseofungin RS, mg						Suppositories KANPROFEM - AK®, mg					
SN	10	9	8	7	6	5	500	450	400	350	300	250
1.1.	0,65	0,65	0,66	0,66	0,65	0,65	0,55	0,56	0,58	0,6	0,63	0,66
1.2.	0,64	0,66	0,64	0,66	0,65	0,64	0,56	0,57	0,59	0,59	0,63	0,65
1.3.	0,66	0,65	0,65	0,66	0,66	0,65	0,55	0,57	0,58	0,59	0,62	0,65
2.1.	0,66	0,64	0,66	0,66	0,65	0,63	0,56	0,56	0,59	0,61	0,62	0,64
2.2.	0,65	0,65	0,65	0,65	0,65	0,64	0,56	0,56	0,59	0,59	0,62	0,65
2.3.	0,65	0,65	0,65	0,66	0,65	0,63	0,55	0,56	0,58	0,59	0,63	0,65
3.1.	0,64	0,65	0,64	0,66	0,66	0,65	0,55	0,56	0,58	0,6	0,62	0,65
3.2.	0,64	0,64	0,65	0,66	0,66	0,65	0,54	0,57	0,58	0,59	0,61	0,66
3.3.	0,65	0,64	0,65	0,64	0,66	0,65	0,55	0,57	0,59	0,6	0,61	0,65
$\bar{X}$	0,65	0,65	0,65	0,66	0,65	0,64	0,55	0,56	0,58	0,60	0,62	0,65

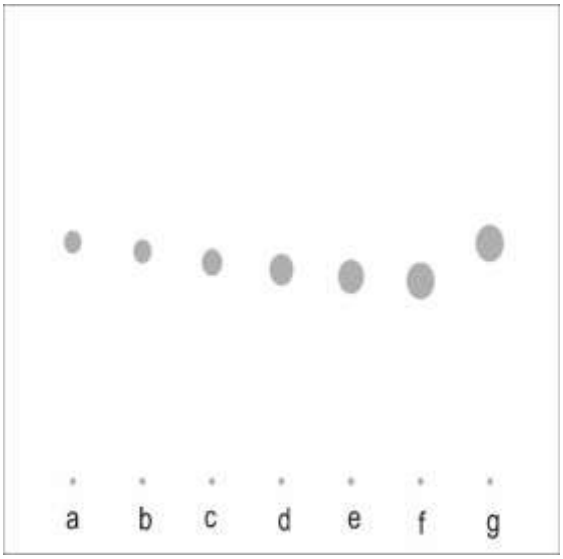

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Figure 2: Chromatogram obtained from the selection of optimal suppository weighed portion: the portion sizes are as follows: a - 250 mg, b – 300 mg, c – 350 mg, d – 400 mg, e – 450 mg, f – 500 mg

Analytical method validation

The following acceptance criteria for Specificity have been proposed:

- there should be no adsorption zones on the placebo chromatogram that have the retardation parameters similar to those of roseofungin adsorption;
- retardation factors (Rf) of roseofungin in the reference solution and the test solution coincide by no less than 98%;
- there are no additional adsorption zones other than the API zone on the chromatogram of the test solution.

In order to conduct a validation assessment, we also examined a placebo solution.

Placebo solution. 0.250 g of the suppository base is placed in a 10 ml volumetric flask, 5 ml of 96% ethanol P is added; the mixture is heated in a water bath at a temperature of 40° C until the suppository base is completely melted, cooled, and the final solution is adjusted to the mark with the same solvent, stirred and filtered.

In order to examine the stability of roseofungin during heating of the test solution to melt the suppository mass, the effect of temperature regime of melting on the API stability was studied. To this end, the solution of Roseofungin RS was heated at 25, 30, 35, 40, 45, 50° C. It was found that the temperature in the examined range did not affect the stability of the roseofungin molecule, since additional adsorption zones did not appear on the chromatograms as a result of thermal exposure (Figure 3).

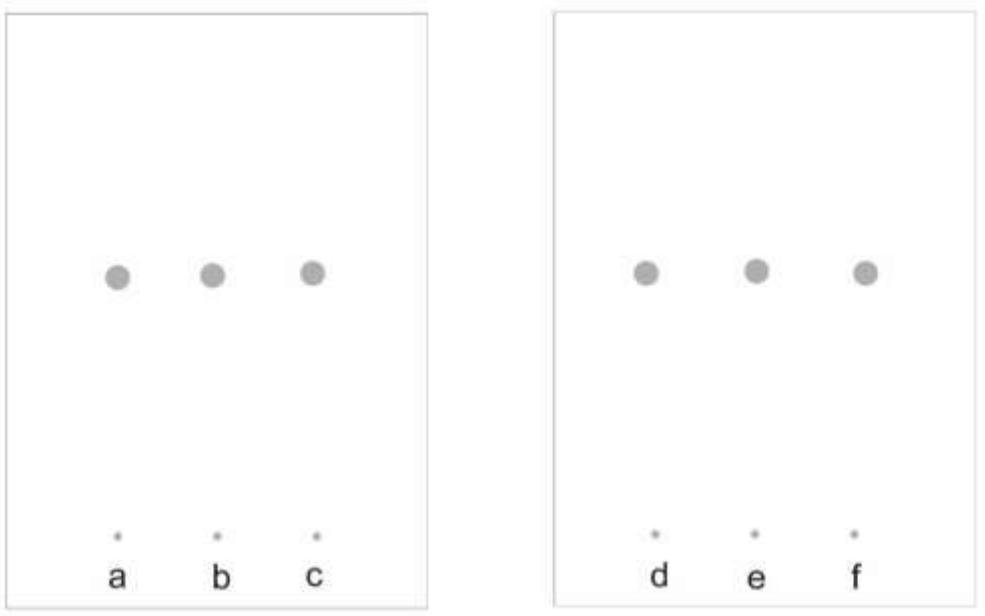


Figure 3: Chromatograms obtained from the study of the effect of temperature regime on roseofungin stability

The results of evaluating the specificity of the analytical method are shown in Table 3 and Figure 4. As can be seen from the data presented in the Table and in the Figure, there is one adsorption zone with yellow fluorescence (Rf values are 0.64 and 0.65, respectively) on the chromatograms of CANPROFEM – AK® suppositories and Roseofungin RS in UV light. No other adsorption zones are observed. The chromatogram of the suppository base has no adsorption zones.

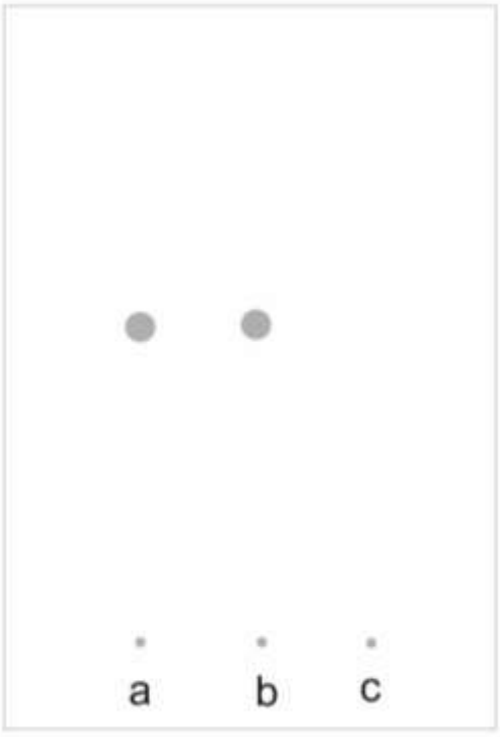


Figure 4: Chromatogram obtained during assessment of specificity of the method for identification of roseofungin: a – Roseofungin RS, b - test solution, c-suppository base

Table 3: Assessment of specificity of the analytical method

	Rf value	
SN	Roseofungin RS	CANPROFEM – AK suppositories
1.1.	0.65	0.65
1.2.	0.65	0.64
1.3.	0.65	0.64
2.1.	0.64	0.65
2.2.	0.64	0.64
2.3.	0.64	0.64
3.1.	0.65	0.64
3.2.	0.65	0.64
3.3.	0.65	0.64
$\overline{X}$	0.65	0.64
$\overline{X}_{sup}/\overline{X}_{RS}$, %	98.46%	

TLC technique is therefore characterized by sufficient specificity and makes it possible to unambiguously evaluate the Roseofungin API in the presence of placebo components

Method 2

To evaluate the suitability of the spectral method for identifying roseofungin, a weighed portion of the drug was examined that was comparable in amount of the substance with a weighed portion of roseofungin in AND for API. The solvent was selected taking into account the solubility of API and suppository base. 96% ethanol was the optimal solvent in this case, in which the substance was completely soluble, and the suppository base did not dissolve. At a temperature of 45° C, the base melted and precipitated upon cooling in the form of a precipitate that is easily filtered. The optimum melting temperature of the suppository mass of 45° C was selected, since at the indicated temperature, as mentioned above, the polyene antibiotic remained stable. The use of heating was due to the need to achieve the maximum release completeness of API from the suppository base. After cooling the obtained extract, filtration was carried out, and the volume of the filtered solution was adjusted to a predetermined value.

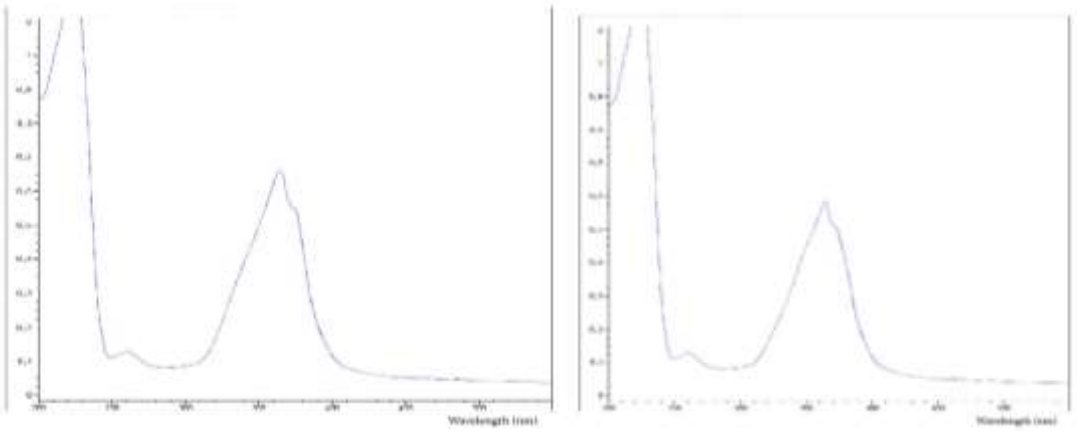
Analytical method validation

Acceptance criteria for the Specificity characteristic

- The UV spectrum of the test solution in the 200-600 nm range should correspond to the UV spectrum of the roseofungin reference solution and have maximum absorbance at a wavelength of 364±2 nm;
- The UV spectra of the placebo solution and 96% ethanol P should not have maximum absorbance corresponding to the maximum absorbance of the roseofungin reference solution;
- The ratio of the absorption of a placebo solution and 96% ethanol P to the absorption of the roseofungin reference solution at a wavelength of 364 nm should be not more than 1.0%.

The results of evaluating the specificity of the spectral technique are shown in Figures 5-6 and in Tables 4-6.

In the 200-600 nm wavelength range, roseofungin has a maximum at λ=364±2 nm. As can be seen from the data in Figure 5 and Table 4, there is a coincidence between the maximum absorbance of the test solution and the reference solution (365 nm). The ratio of the absorption of a placebo solution and 96% ethanol P to the absorption of the roseofungin reference solution at a wavelength of 365 nm is 0.52%, which is less than 1.0% and therefore meets the acceptance criterion.

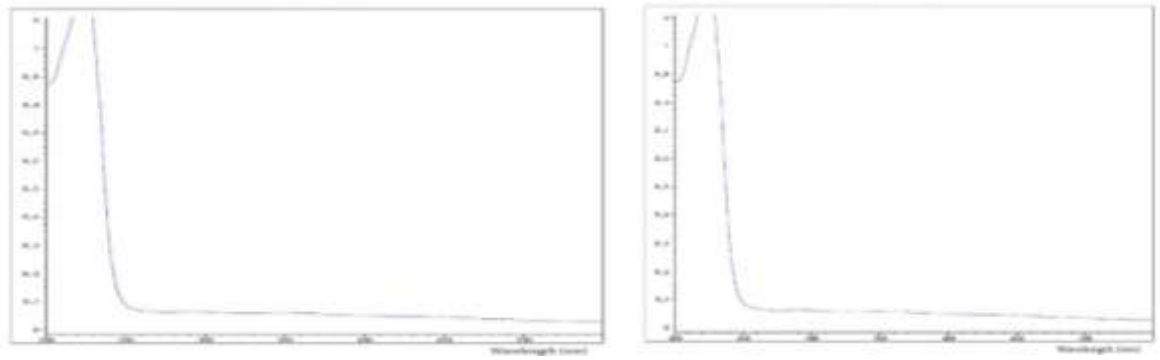


a b

Figure 5: Spectra of the test solution (a) and reference standard solution (b)

Table 4: The results of determining maximum absorbance of the reference and test solutions

Name of solution	Wavelength at which maximum absorbance is observed, nm
Reference solution	365
	365
	365
Test solution	366
	365
	365



a b

Figure 6: a - UV spectrum of placebo solution, b - UV spectrum of *96 % ethanol P*

Table 5: The results of determining the average optical density of 96% *ethanol P*, placebo solution, and reference solution at a wavelength of 365 nm

Name of solution	Average value of **A** (ODU)
96 % ethanol P	0.0002
Placebo solution	0.003
Reference solution	0.574

Table 6: Optical density of the reference solution

Name of solution	**A** (ODU)	Average value of **A** (ODU)
Reference solution	0.572	0.574
	0.576	
	0.575	

We can thus conclude that the proposed method makes it possible to precisely and selectively determine roseofungin in suppositories in the presence of placebo ingredients.

CONCLUSION

The suitability assessment was carried out and the validity of two analytical methods for identifying roseofungin by TLC and spectrophotometry in the ultraviolet region in CANPROFEM - AK suppositories was proved. The optimal weighed portion of CANPROFEM - AK (250 mg for TLC, 50 mg for UV spectrophotometry) was selected. The specificity of the proposed analytical methods was proved and acceptance criteria were established. The methods are acceptable for inclusion in the draft analytical normative document to determine the quality of the finished product, as well as quality specifications for intermediates in the production process.

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