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Professional paper

Validation of analytical method for determination of microbiological quality of antifungal shampoo containing 2% ketoconazole: Enumeration test and tests for specified microorganisms (Method suitability test)

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Abstract

Microbiological quality screening of non - sterile product is conducted by suitable enumeration tests and tests for absence of specified microorganisms. The European Pharmacopoeia offers several methods for conducting these tests.

The suitability of a compendial microbiological method must be demonstrated to ensure accuracy and reliability of test results. The work presented here had the purpose of creating a validated and reliable method for screening the microbiological quality of antifungal shampoo containing 2% ketoconazole. Method suitability tests for the parameters: Total aerobic microbial count (TAMC), Total yeast and mold count (TYMC), absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were determined according to chapters 2.6.12. Microbial examination of non – sterile products: microbial enumeration tests and 2.6.13. Microbial examination of non – sterile products: test for specified microorganisms. The membrane filtration method was chosen for TAMC and TYMC.

The criteria for method suitability of the counting methods TAMC and TYMC – a mean count of each of the microorganisms differing by a factor not greater than 2 from the value of the control in the absence of the product (recovery rate 50% - 150% of the test group with product in comparison with the positive control group) was obtained. The criteria of growth on selective media for *P. aeruginosa* and *S. aureus* were also obtained.

Key words: microbiological quality, method suitability, antifungal shampoo.

Introduction

Pyrogenic Ketoconazole 1-[(52RS,54SR)-52-(2,4-Dichlorophenyl)-3-oxa-1(1)-piperazina-7(1)-imidazola-5(2,4)-[1,3]dioxolana-2(1,4)-benzenaheptaphan-11-yl]ethan-1-one, (European Pharmacopoeia, Monograph 04/2024:0921, 2024) (Fig.1), an anti-fungal agent, is often incorporated in several pharmaceutical forms and in shampoo formulation. It is known to be effective against fungal infection on the scalp (Staub et al., 2005). Ketoconazole belongs to the imidazole antifungal class of medications (Sinawe and Casadesus, 2023). Topical ketoconazole appears to be an effective therapy for seborrheic dermatitis of the scalp, and more suitable for long-term treatment than the oral preparation (Carr et al., 1987). Ketoconazole 2% shampoo is highly effective, not only in clearing scalp seborrheic dermatitis and dandruff,

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but also in preventing relapse of the disease when used prophylactically once weekly (Peter and Richarz, 1995). In microbiological terms, pharmaceutical products can be divided into two groups: sterile and non-sterile (Ratajczak et al., 2014). This antifungal shampoo falls under the category non - sterile products. The microbiological acceptance criteria of non-sterile drug products are generally based on the tripartite harmonized informational chapters of the European Pharmacopoeia, United States Pharmacopoeia and Japanese Pharmacopoeia and are composed of a quantitative total aerobic microbial count and total yeasts/molds count and the absence of specified microorganisms (Roesti, 2019). Non-sterile drugs must satisfy the appropriate microbiological purity criteria which are included in pharmacopoeial monographs (Ratajczak et al., 2014). Chapter 5.1.4 of the European Pharmacopoeia: Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use, contains proposed microbial quality specifications based on the route of administration. Acceptance criteria for microbiological quality of products for cutaneous use for total aerobic microbial count (TAMC) and the total combined yeasts/molds count (TYMC) as well as the absence of specified microorganisms according to European Pharmacopoeia 5.1.4.-1 are shown in Table 1 (European Pharmacopoeia, 5.1.4, 01/2021:50104, 2024, United States Pharmacopoeia, 2020).

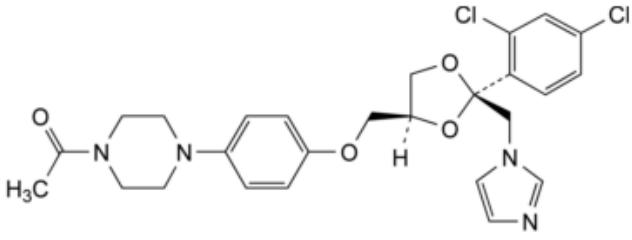

Fig. 1. Structural formulae of Ketoconazole

Aqueous, multiple-use, non-sterile drug products are susceptible to microbial contamination during their shelf life (Cundell, 2015). The presence of certain micro-organisms in non-sterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient (Clonz, 2009). The effects range from mild changes (e.g., discoloration, texture alteration) to severe effects (e.g., changes in activities, toxicity) (Dao et al., 2017). In order to ensure the microbial quality of drug products, a scientifically sound quality control testing program must be implemented (Clonz, 2009). To achieve the quality objective, it is necessary to control all stages of drugs, which covers all matters, which individually or collectively influence the quality of a product, including raw materials, the manufacturing process and the evaluation of finished product. One of the control stages is the assessment of microbiological quality of medicinal products (Tyski, 2011). Where microbial testing of product is carried out, the method should be validated to confirm that the product does not influence the recovery of microorganisms (EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use, Annex 15, 2015).

The methodologies described in the compendia for examination of microbial quality of non - sterile products include quantitative (bioburden) and qualitative tests for specified organisms. (Clonz, 2009). Chapter 2.6.12 of the European Pharmacopoeia provides tests for the quantitative estimation of aerobic mesophilic bacteria (TAMC) and fungi (TYMC) that might be present in pharmaceutical products. These tests are designed primarily to verify whether a sample complies with the established specification for microbial quality. The TAMC and TYMC tests can be performed using membrane filtration, pour-plate, or spread-plate techniques. The choice of method, which must be qualified, is based on the type of product to be tested and product specifications and must be validated/qualified prior to use. The tests described in the European Pharmacopoeia section 2.6.13 are designed to determine whether a non - sterile product complies with established specifications for absence of specified organisms. The ability of the compendial method to detect microorganisms that might be present in the product being tested must be established. (Clontz, 2009).

Method suitability studies should be performed prior to the examination of a product for its microbial content. If a product possesses antimicrobial properties because of the presence of a specific preservative or due to its formulation, the antimicrobial property must be neutralized in order to recover viable microorganisms by means of dilution, washing, filtration, and/or chemical inactivation to ensure adequate recovery of viable microorganisms. Taking into consideration the antifungal activity of the active pharmaceutical ingredient – ketoconazole, membrane filtration was chosen for TAMC and TYMC method. Filtration is listed as a suitable means to remove antimicrobial agents from sample preparations. This is because during filtration the sample is filtered along with any chemical inhibitors and all that is left on the membrane filter are microbial cells that might be present in the product (Clontz, 2009). Adequate recovery of the test organisms confirms the suitability of the test method. Failure to recover the test organisms necessitates specific modifications to the test procedure.

The aim of this study was to determine the suitability of the chosen method for testing microbiological quality of antifungal shampoo containing 2% ketoconazole. The purpose of this test was to establish a reliable method for routine testing of the microbiological purity of the product.

Table 1. Acceptance criteria for microbiological quality of non-sterile dosage forms - cutaneous use

Route of administration	TAMC (CFU/g)	TYMC (CFU/g)	Specified micro-organisms
Cutaneous use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)

Material and methods

Materials

An antifungal shampoo product containing 2% ketoconazole was used as a sample for conducting the method suitability test. Three commercial batches were used for the purpose of determining the suitability for each cycle. The following media and reagents manufactured in Biomerieux, France were used: Tryptic soy agar - non – selective medium used for the cultivation and enumeration of bacteria, Saboraud dextrose agar agar - non – selective medium used for the cultivation and enumeration of yeast and mould, Buffered peptone water (Peptone salt broth) used for preparation of initial suspension and subsequent dilutions of the product as well as preparation of microbial suspension, Neutralizing buffered solution Eugon used for enrichment of microorganisms, Mannitol salt agar for isolation of *Staphylococcus aureus*, Cetrimide agar for isolation of *Pseudomonas aeruginosa*. Nitrocellulose membrane filter, 0,2µm (Sartorius, Germany) was used for filtration of the samples.

The following test microorganisms manufactured in Microbiologisc, USA were used: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ssp. *spizizenii* ATCC 6633, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404.

The following laboratory equipment was used: Biosafety cabinet, class II (Telstar, Spain), Laboratory incubators set at 33 ºC (Binder, Germany) and 25ºC (Panasonic, Japan), Membrane filtration system (Satrorius, Germany), Vacuum pump (Satrorius, Germany), Bunsen burner (Integra, USA), High pressure autoclave (Panasonic, Japan), Laboratory balance (Satrorius, Germany), Sterile forceps (Iso Lab, Germany), Sterile individually wrapped pipets (Thermo Fisher, USA).

Methods

The method suitability tests were obtained according to the European Pharmacopoeia chapters 2.6.12 Microbiological examination of non-sterile products: microbial enumeration tests for the TAMC and TYMC parameters and 2.6.13 Microbiological examination of non-sterile products: test for specified micro-organisms for the parameters absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Total aerobic microbial count (TAMC), total yeast and mold count (TYMC)

Screening of antimicrobial properties of the formulation was the initial step of the method suitability test for the counting methods TAMC and TYMC. If present, they need to be neutralized before proceeding with further stages of the test. The screening was conducted by inoculating initial suspension 1:10 of the product in peptone water with less than 100 colony forming units (CFU) of the test microorganisms (Table 2). There should be visible growth in the inoculated tubes.

It was determined that the product has antimicrobial properties due to the inhibition of the growth of: *Bacillus spizizenii, Candida albicans* and *Aspergillus brasiliensis* (Table 3). Then, the screening of antimicrobial properties was conducted on the subsequent decimal dilution 1:100. A slight inhibition of *Candida albicans* was still present. Therefore, another means of inhibition of the antimicrobial properties of the formulation was included - the use of membrane filtration technique. Given the fact that most, if not all, product material will be filtered through the membrane filter, there is minimum product interference, if any (Clontz, 2009). This fact relies on the physical retention of microorganisms on the membrane filters and the assumption that antimicrobial agents that might be present in the sample will pass through the filter into the filtrate. This theoretical assumption was confirmed with the results presented in Table 3. The results show the satisfactory absence of antimicrobial properties.

After the initial step of screening of antimicrobial properties, method suitability tests were conducted by preparing two test groups for each of the parameters TAMC and TYMC: test group with product (1:100) and test group without product (peptone water). Each group was inoculated with low count suspension of each of the test microorganisms (approximately 100 CFU per plate) (Table 2). The test group without product (positive control group) was used for determination of the referent microorganism count.

100 mL of each of the test groups were filtered through a membrane filter. For the TAMC parameter test the filters were placed on Casein Soya Bean agar. Negative control was prepared by simply filtering 100 mL of peptone water and placing the filter on Casein Soya Bean agar. All the agar plates were incubated at 33 ºC for the duration of three days.

For the TYMC parameter the filters were placed on

saboraud dextrose agar. Negative control was prepared by filtering 100 mL of peptone water and placing the filter on Saboraud dextrose agar. All the agar plates were incubated at 25 ºC for the duration of five days.

At the end of the incubation period, CFU on all the agar plates were counted and recovery rate was calculated using the equation r = b/a *100, where r = recovery rate, a = CFU count of the positive control test group, b = CFU count of the test group with product.

The procedure was conducted in three cycles for each test microorganism.

Absence of specified microorganisms

For the method suitability test for absence of specified microorganisms three test groups were prepared: test group with product, test group without product and test group without neutralizers. The test groups were prepared as follows:

Test group with product: 10 mL of the initial suspension (1:10 product to peptone water) was transferred in 90 mL enrichment broth.

Test group without product: 10 mL of peptone water was transferred into 90 mL enrichment broth. This test group was used for determination of the referent microorganism growth.

Test group without neutralizers: 100mL peptone water. This test group served the purpose of confirming that the neutralizing agents contained in the enrichment broth are not toxic to the test microorganisms.

The test groups were inoculated with low microbial count of the test microorganisms *Pseudomonas aeruginosa* and *Staphylococcus aureus*, approximately 100 CFU and incubated at 33 ºC for the period of 24 hours.

Additionally, negative control group and test group without test microorganism were prepared.

Test group without test microorganism: 10 mL of the initial suspension (1:10 product to peptone water) was transferred in 90 mL enrichment broth. This test group served the purpose of confirming that the results from the other test groups are not false positive.

Negative control: 10 mL peptone water was added to 90 mL of enrichment broth. It is prepared for confirmation of the sterility of the media and used equipment.

These test groups were also incubated at 33 ºC for the period of 24 hours.

Subculture of 0,1 mL of all of the test groups was made on Cetrimide agar for the *Pseudomonas aeruginosa* test and Mannitol Salt agar for the *Staphylococcus aureus* test: The agar plates were incubated at 33 ºC for the period of 24 hours. Comparison of growth was being observed.

Table 2. Media and test conditions for method suitability - test group with product

Microorganism	TAMC	TYMC
Staphylococcus aureus	Casein Soya Bean Digest agar ≤100 CFU 30˚C – 35˚C 3 – 5 days	N/A
Pseudomonas aeruginosa	Casein Soya Bean Digest agar ≤100 CFU 30˚C – 35˚C 3 – 5 days	N/A
Bacillus spizizenii	Casein Soya Bean Digest agar ≤100 CFU 30˚C – 35˚C 3 – 5 days	N/A
Candida albicans	Casein Soya Bean Digest agar ≤100 CFU 30˚C – 35˚C 3 – 5 days	Sabouraud dextrose agar ≤100 CFU 20˚C – 25˚C 5 – 7 days
Aspergillus brasiliensis	Casein Soya Bean Digest agar ≤100 CFU 30˚C – 35˚C 3 – 5 days	Sabouraud dextrose agar ≤100 CFU 20˚C – 25˚C 5 – 7 days

Results and discussion

Total aerobic microbial count (TAMC), total yeast and mold count (TYMC)

The results obtained for the tests of each microorganism in each of the three cycles for TAMC and TYMC parameters are displayed in table 4.

The plates of the negative control group did not have any growth.

Absence of specified microorganisms

Clear growth of green colonies was detected on the Cetrimide agar plates of the test groups inoculated with *Pseudomonas aeruginosa*. Clear growth of yellow colonies

surrounded with yellow zone was detected on the Mannitol salt agar plates of the test groups inoculated with *Staphylococcus aureus*. The negative control and the test group without test microorganisms did not have any growth. The results obtained for the tests of each microorganism in each of the three cycles are displayed in Table 5.

Table 3. Screening antimicrobial properties of antifungal shampoo

Test microorganism	Media	Growth without product	Growth with product
	1:10 dilution		
Bacillus spizizenii	Casein Soya Bean Digest agar	+	-
Staphylococcus aureus	Casein Soya Bean Digest agar	+	+
Pseudomonas aeruginosa	Casein Soya Bean Digest agar	+	+
Candida albicans	Casein Soya Bean Digest agar	+	-
Candida albicans	Sabouraud dextrose agar	+	-
Aspergillus brasiliensis	Casein Soya Bean Digest agar	+	-
Aspergillus brasiliensis	Sabouraud dextrose agar	+	-
	1:100 dilution after filtration		
Bacillus spizizenii	Casein Soya Bean Digest agar	+	+
Staphylococcus aureus	Casein Soya Bean Digest agar	+	+
Pseudomonas aeruginosa	Casein Soya Bean Digest agar	+	+
Candida albicans	Casein Soya Bean Digest agar	+	+
Candida albicans	Sabouraud dextrose agar	+	+
Aspergillus brasiliensis	Casein Soya Bean Digest agar	+	+
Aspergillus brasiliensis	Sabouraud dextrose agar	+	+

+ = good growth, ± = week growth, - = no growth

Table 4. Recovery of microorganisms for TAMC and TYMC

Test microorganism	$r_{cycle\ I}$ (%)	$r_{cycle\ II}$ (%)	$r_{cycle\ III}$ (%)
	TAMC		
Bacillus spizizenii	79	71	81
Staphylococcus aureus	97	91	83
Pseudomonas aeruginosa	97	89	90
Candida albicans	78	73	79
Aspergillus brasiliensis	98	86	91
	TYMC		
Candida albicans	84	70	72
Aspergillus brasiliensis	74	86	89

Table 5. Method suitability test for absence of Staphylococcus aureus and Pseudomonas aeruginosa

		Staphylococcus aureus			
	Media	Test group with product	Test group without product	Test group without neutralizers	Test group without test microorganism
Cycle I, Inoculum 105 CFU	Test solution	Visible growth	Visible growth	Visible growth	No growth
	[1]MSA	yellow colonies surrounded by a yellow zone	yellow colonies surrounded by a yellow zone	yellow colonies surrounded by a yellow zone	No growth
Cycle II, Inoculum 97 CFU	Test solution	Visible growth	Visible growth	Visible growth	No growth
	[1]MSA	yellow colonies surrounded by a yellow zone	yellow colonies surrounded by a yellow zone	yellow colonies surrounded by a yellow zone	No growth
Cycle III, Inoculum 88 CFU	Test solution	Visible growth	Visible growth	Visible growth	No growth
	[1]MSA	yellow colonies surrounded by a yellow zone	yellow colonies surrounded by a yellow zone	yellow colonies surrounded by a yellow zone	No growth
		Pseudomonas aeruginosa			
Cycle I, Inoculum 101 CFU	Test solution	Visible growth	Visible growth	Visible growth	No growth
	Cetrimide agar	Green colonies	Green colonies	Green colonies	No growth
Cycle II, Inoculum 97 CFU	Test solution	Visible growth	Visible growth	Visible growth	No growth
	Cetrimide agar	Green colonies	Green colonies	Green colonies	No growth
Cycle III, Inoculum 91 CFU	Test solution	Visible growth	Visible growth	Visible growth	No growth
	Cetrimide agar	Green colonies	Green colonies	Green colonies	No growth

[1] Mannitol Salt Agar

Conclusion

Microbiological quality of shampoo containing 2% ketoconazole is monitored by screening the TAMC and TYMC parameters as well as absence *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The counting parameters TAMC and TYMC are tested by using the membrane filtration technique after 1:100 dilution of the

product. The results from the method suitability test showed that the acceptability factor for all test microorganisms in each of the three performed cycles of three industrial batches of the product does not exceed the factor 2 and complies with the requirements of the European Pharmacopoeia. Therefore, this technique is chosen for routine testing of the TAMC and TYMC parameters of this preparation.

For the absence of Staphylococcus *aureus* and *Pseudomonas aeruginosa*, the criteria of the European Pharmacopoeia – visible growth of the inoculated groups and absence of growth in the negative group and the group without microorganisms was met. Therefore, it can be used for routine testing of the product.

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Резиме

Соодветност на аналитички метод за определување на микробиолошка чистота на антифунгален шампон кој содржи 2% кетоконазол: тестови на броење и тестови за отсуство на специфицирани микроорганизми

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Клучни зборови: микробиолошки квалитет, соодветност на метод, антифунгален шампон

Микробиолошкиот квалитет на нестерилен производ се определува преку спроведување соодветни тестови за броење на микроорганизми и отсуство на специфицирани микроорганизми. Во Европската Фармакопеја се наведени неколку методи за изведување на овие тестови. Нивната соодветност мора да се докаже со цел добивање на точни и веродостојни резултати при рутинска анализа. Целта на овој труд е добивање на валидиран и веродостоен метод за проверка на микробиолошкиот квалитет на антифунгален шампон кој содржи 2% кетоконазол. Соодветноста на параметрите за броење: вкупен број аеробни микроорганизми и вкупен број квасци и мувли, како и за параметрите отсуство на специфицираните микроорганизми: *Staphylococcus aureus* и *Pseudomonas aeruginosa*, беше спроведена според поглавјата 2.6.12. и 2.6.13 на Европската Фармакопеја. За параметрите вкупен број аеробни микроорганизми и вкупен број квасци и мувли беше одбрана техника на мембранска филтрација. Критериумот за соодветност – фактор на прифатливост помал од 2 беше исполнет. Критериумот за раст на специфицирани микроорганизми беше исто така исполнет.