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Analytical method development and validation of antifungal drugs in updated ointment formulation using UV spectroscopy and RP-HPLC

RETHINA KARUPPIAHYA[1], SUBA GEETHA ARUNACHALAM[1], SARAVANAN VENKATTAPURAM SAMPATH[1], SAMBATHKUMAR RAMANATHAN[1], ANANDA THANGADURAI SUBRAMANIAM[2], RAVIKUMAR RAMASAMY[1], ANUPRINCY PAULMURUGAN[1] AND JAMBULINGAM MUNUSAMY[1]*

[1]*Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, Tamil Nadu, India, and* [2]*Department of Pharmaceutical Analysis, JKKN College of Pharmacy, Kumarapalayam, Namakkal District, Tamil Nadu, India.*

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Abstract: A precise, simple, and validated Reverse-phase high-performance liquid chromatography (RP-HPLC) method alongside spectrophotometric analysis has been established for the simultaneous quantification of clobetasol propionate, miconazole nitrate, and salicylic acid in an updated ointment formulation. The impact of organic modifiers on the retention of the target compounds was assessed. The chromatographic analysis was conducted using a simple low-pressure gradient method with UV detection at 282 nm on a C18 column (5 µm, 4.6 × 250 mm HSS). The mobile phase consisted of a mixture of methanol, acetonitrile, and tetra ethylamine acetate buffer at pH 4, adjusted with acetic acid in a 40:40:20 v/v ratio, at a flow rate of 1.2 mL min^{-1}. The method demonstrated linearity in the 5-15 µg mL^{-1} concentration range for clobetasol propionate, 30-90 µg mL^{-1} for miconazole nitrate, and 15-45 µg mL^{-1} for salicylic acid. The limits of detection and quantification were determined to be 1.49 µg mL^{-1} and 4.53 µg mL^{-1} for clobetasol propionate, 8.72 µg mL^{-1} and 26.43 µg mL^{-1} for miconazole nitrate, and 3.37 µg mL^{-1} and 10.22 µg mL^{-1} for salicylic acid, respectively. The recoveries for drugs in formulation range from 95 % to 99 %.

Keywords: clobetasol propionate; miconazole nitrate; salicylic acid; high-performance liquid chromatography; UV spectroscopy.

INTRODUCTION

Fungal infections pose a notable challenge to skin wellness, impacting people across all age groups. From irritating nail infections to bothersome conditions such as jock itch, athlete's foot, and ringworm, the array of fungal ailments calls for

* Corresponding author. E-mail: jambulingam48@gmail.com
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efficient treatment. If neglected, these infections can progress to more serious health issues, underscoring the importance of prompt intervention.[1]

Clobetasol propionate (Fig S2, Supp. material) chemically designated as [(8S,9R,10S,11S,13S,14S,16S,17R)-17-(2-chloroacetyl)-9-fluoro-11-hydroxy-10,13,16 trimethyl-3-oxo-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] propanoate is a potent topical glucocorticoid.[2] It is an analog of prednisolone, acts by binding glucocorticoid receptors in the cytoplasm, and activates gene transcription via the glucocorticoid receptor. This produces anti-inflammatory proteins and limits the activity of inflammatory mediators.[3] Thus increasing the creation of phospholipase A2 suppressor proteins, which regulate the release of arachidonic acid, an inflammation precursor. Mainly used in the treatment of psoriasis, eczema, contact dermatitis, and lichen planus.[4]

Miconazole nitrate (Fig S3, Supp. material) chemically named as 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl) methoxy] ethyl] imidazole; nitric acid and classified as an imidazole antifungal agent.[5,6] Miconazole nitrate works by inhibiting the synthesis of ergosterol, a crucial component of fungal cell membranes thus effective in the treatment of many fungal infections.[7,8]

Salicylic acid (Fig S1, Supp. material) chemically named 2-hydroxybenzoic acid works as a keratolytic agent, it helps to break down and exfoliate dead skin cells.[9] This property makes it effective in treating conditions where there is an abnormal buildup of skin cells, such as acne, psoriasis, and keratosis pilaris. Additionally, its anti-inflammatory and antimicrobial properties contribute to its usefulness in addressing various skin issues.[10,11]

Various formulations of clobetasol propionate, salicylic acid, and miconazole nitrate are available for topical use, encompassing ointments, creams, gels, foams, cosmetic products, and acne treatment preparations.[3,4,9,12]

A review of the literature showed that clobetasol propionate was determined using a variety of analytical techniques, including TLC-densitometric method,[13] RP-HPLC,[2,11,8] UV spectrophotometry,[14] and UPLC-MS/MS.[15] Spectrofluorometric,[16] RP-HPLC,[5,7,8] UV Spectroscopy,[7] UPLC,[5] capillary zone electrophoresis,[17] LC-MS/MS[18] and Gas Chromatography - Flame Ionization Detector (GC-FID)[19] were among the techniques used to analyze Miconazole. LC-Tandem mass spectrometry,[9] liquid chromatography with solid phase extraction,[20] TLC-densitometric techniques,[21] LC-MS/MS,[9] SPE-UHPLC-MS/MS,[22] UPLCMS/MS,[9] LC-MS/MS,[9] GC-MS,[9] spectrofluorimetry,[9] UV spectrometry,[9] Liquid-Liquid Extraction- HPLC,[9] and RP-HPLC[9,11] were among the methods used to analyze salicylic acid.

This article presents a comprehensive approach to treating skin conditions by combining antifungal and corticosteroid agents in topical formulation namely an ointment. This synergistic strategy targets both fungal infections and inflammation, further enhanced by the addition of keratolytic agents to promote

skin cell turnover and absorption of active ingredients. Utilizing a polyethylene glycol base ensures optimal penetration and patient compliance, resulting in efficient relief and accelerated recovery across various dermatological conditions.[23]

Furthermore, the article addresses a gap in analytical methods by proposing the development of an RP-HPLC and UV spectroscopy method to determine the percentage of drugs in the ointment, complementing existing literature on salicylic acid with clobetasol propionate,[11] and miconazole nitrate with clobetasol propionate.[8] The integration of these analytical techniques promises a more comprehensive understanding and evaluation of multifaceted formulations in dermatological care. The proposed method is reproducible, reliable, and economical and permits the analysis of more samples in a short period of 15 minutes.

EXPERIMENTAL

Chemicals and reagent

Clobetasol propionate and miconazole nitrate were obtained as a gift sample from Ajanta Pharma, Hyderabad. The following analytical grade reagents like salicylic acid, acetonitrile, and HPLC water were obtained from Merck (Vikhroli, Mumbai). Acetic acid, triethylamine, methanol, polyethylene glycol (PEG) 400, and polyethylene glycol (PEG) 4000 were obtained from Lobachemei Pvt. Limited, Mumbai.

Instrumentation:

Spectroscopic analysis was performed using a Shimadzu UV-1900i double-beam spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells and data produced by the program Lab Solution version (1.13), data acquisition was performed using MATLAB (6.2.1) version.

RP-HPLC was performed using a Shimadzu P-series PDA liquid chromatography system (Shimadzu, Japan) consisting of a column oven (CTO-10AS VP), a pump (LC-20ATD), a manual injector and a degasser (DGU20A3R/20A5R) and SPD 40 (Photodiode Array Detector-UV-Vis Detector) was used for UV detection. Then the chromatographic conditions were controlled by a software package named Lab Solutions.

Different column packing and mobile phases were tested to develop and formulate a method for the simultaneous determination of clobetasol propionate, miconazole nitrate, and salicylic acid in ointment concerning the shape of peaks in the corresponding chromatogram. The final choice of the stationary phase consists of a C18 column (5µm, 4.6 x 250 mm HSS), which shows great resolution and run time. The degassing of the mobile phase was performed by passing through a 0.45 µm membrane filter and sonicator. A flow rate of 1.2 mL min^{-1} was used for the separation, and an internal standard with UV detection at 282 nm was used as the detector wavelength, a column oven temperature of 30 °C was used with the injection volume of 20 µL.

Preparation of topical formulation:

Weigh about 15g of PEG 4000 and 31 mL of PEG 400, then stir until the mixture achieves a semisolid consistency at room temperature. Then use the fusion technique to add the appropriate quantities of clobetasol propionate, miconazole nitrate, and salicylic acid in

succession, ensuring thorough incorporation and a uniform distribution.[23] (Table SI, Supp. material)

Preparation of standards and sample stock solution (RP-HPLC and UV-visible spectroscopy)

A standard stock solution of clobetasol propionate, miconazole nitrate, and salicylic acid was prepared at a concentration of (1000 µg mL^{-1}). From this standard stock solution, prepare the aliquots of different concentrations by suitable dilutions using ethanol for spectroscopic measurements. In the same way sample solution was prepared for RP-HPLC using the diluent (acetonitrile: methanol at 50:50% v/v).

A combined standard stock solution was prepared by accurately weighing and transferring 10mg of clobetasol propionate, 60mg of miconazole nitrate, and 30mg of salicylic acid in a 100 mL volumetric flask. Add the required amount of diluent, and sonicate to dissolve and make up the volume to 100 mL which may contain 100 µg mL^{-1} of clobetasol propionate, 600 µg mL^{-1} of Miconazole nitrate, 300 µg mL^{-1} of salicylic acid. The final concentration was made to 50 µg mL^{-1},75 µg mL^{-1},100 µg mL^{-1},125 µg mL^{-1} and 150 µg mL^{-1} respectively by diluent. Filter the final solutions using a membrane filter 0.45 µm of pore size.

Sample stock solution was prepared by accurately Weighing 2g of antifungal topical formulation which contains 0.01g clobetasol propionate, 0.6 g of salicylic acid, 0.039 g of miconazole nitrate in a 100 mL volumetric flask, add 50mL of ethanol and sonicate to dissolve. After lightly warming the solution to dissolve completely make up the volume with solvent ethanol for spectroscopic measurements (1000µg mL^{-1}). From the sample stock solution, the final concentration was made to 20 µg mL^{-1} for spectroscopic analysis. In the same way sample solution was prepared for RP-HPLC using the diluent (acetonitrile: methanol at 50:50% v/v)

Spectrophotometric analysis

Wavelength selection

From the standard stock, prepare 20 µg mL^{-1} of the solution and scan from 200-400 nm. The maximum absorption was noted for clobetasol propionate at 239 nm, miconazole nitrate at 216 nm, and salicylic acid at 303 nm respectively. (Fig S4, Supp. material).

MATLAB analysis:

To achieve a concentration of 20 µg mL^{-1} for each analyte, dilutions are prepared from the standard stock solution. A 3*3 matrix was constructed using different wavelengths selected according to the maximum absorption of the compound in the UV spectrum (illustrated in Figure 1), and MATLAB was employed to calculate the amount of drugs in the formulation.

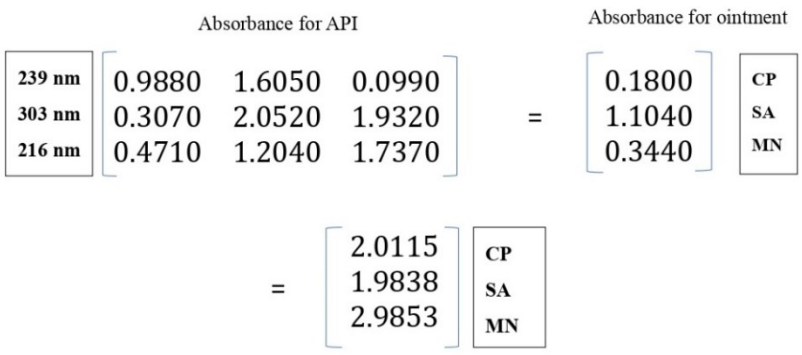

Fig 1. Shows a 3*3 Matrix used for calculating drug content via MATLAB

Chromatographic analysis:

Linearity range: Typical linear concentrations were constructed with five samples of combined stock solution. The linearity range was 5 – 15 µg mL^{-1} for clobetasol propionate, 30 - 90 µg mL^{-1} for miconazole nitrate, and 15 - 45 µg mL^{-1} for salicylic acid. The linear line was obtained by plotting the ratios of the peak areas of clobetasol propionate, miconazole nitrate, and salicylic acid versus their concentration in µg mL^{-1}. The regression equations were calculated by the Excel sheet.

Accuracy samples were prepared at 80 %, 100 %, and 120 % in the same way as linearity samples.

RESULTS AND DISCUSSION

Several evaluation parameters of formulated ointment were investigated and confirmed that the formulation passes the evaluation test for topical application. Evaluation of physical parameters revealed that a white, uniform and consistent preparation with good homogeneity was found to have a pH of 4.9 ± 0.06, indicating their potential suitability for dermatological care. The viscosity of formulation was performed at different speeds and values to adhere to the acceptance criteria for skin application with 822000 cps.

The spreadability of the new formulation was determined to be 17 cm^2/s which indicates the moderate to high spreadability rate. Drug content is measured using liquid chromatography with a high % recovery of 96 %, 99 %, and 95 % for clobetasol propionate, miconazole nitrate, and salicylic acid respectively. (Table SII, Supp. material)

Spectrophotometric analysis using MATLAB

Using a 3*3 matrix in MATLAB software, the drug concentration in an ointment was calculated. Each entry in the matrix indicates the amount of different drugs that are present. By using these measurements from MATLAB in conjunction with a series of calculations, the amount of drug in the topical formulation is calculated to be 98.6 % for clobetasol propionate, 92.2 % for miconazole nitrate, and 109.70 % for salicylic acid. (Table SIII, Supp. material)

Method development:

Several variables of the RP-HPLC method concerning their effect on the separation of clobetasol propionate, miconazole nitrate, and salicylic acid were investigated. In comprehensive preliminary trials, a series of mobile phases with different pH in combination with different organic solvents were tested.

The chromatographic conditions for the simultaneous quantification of clobetasol propionate, salicylic acid, and miconazole nitrate using RP-HPLC were patiently constructed after experimenting with several mobile phases, solvent-buffer ratios, and pH conditions. The presence of organic modifiers in the mobile phase has a significant effect on the retention of analytes, which are mostly adsorbed onto the stationary phase. Systematic experimentation revealed that a mobile phase containing methanol, acetonitrile, and tetra ethylamine acetate buffer

at pH 4, adjusted with acetic acid in a 40:40:20 v/v ratio, provided optimal retention times for the target compounds, all in less than 15 minutes.

Given the solubility of all drugs in a methanol and acetonitrile mixture, initial trials utilized these solvents. However, the inclusion of a buffer became necessary due to the weak acid nature of salicylic acid and the weak base properties of miconazole, demanding a buffer with a pKa value surpassing that of both drugs' pH levels. Acetate buffer was thus incorporated into the mobile phase in varying compositions. Adjustments were meticulously made to pH, temperature, and flow rate to perfect the method, ensuring precision, accuracy, and suitability. Which revealed better system suitability parameters. (Table SIV, Supp. material)

Method validation

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) provides guidelines for the validation of analytical procedures, commonly known as the ICH Q2(R1) guideline, which is used for validation of the optimized method.[24]

Linearity

The linearity was validated on samples of standard clobetasol propionate, miconazole nitrate, and salicylic acid at five different concentrations (5-15 µg mL^{-1}, 30-90 µg mL^{-1}, and 15-45 µg mL^{-1}). The regression equations for clobetasol propionate, miconazole nitrate, and salicylic acid were: $y = 4063.3x + 1899.1$, $y = 1181.7x + 1196.3$, and $y = 19107x + 20494$. The correlation coefficients were 0.9922, 0.9957, and 0.9954, respectively, where x represents concentration in µg mL^{-1}, y represents HPLC peak area, which was automatically tracked by an integrator of the HPLC equipment, and R implies the correlation coefficient. The data entry and analysis were successfully carried out on a personal computer using Microsoft Excel (Office Home & Student 2021 Microsoft Co., Redmond, USA, 2021). (Table SV and Fig. S5, Supp. material)

Precision

Triplicate samples of each drug were prepared and analyzed using the proposed RP-HPLC technique on the same day and three days apart. The related coefficients of variation were then calculated. Table II summarizes the method's intra- and inter-day variability. These findings support the method's high accuracy and consistency, both within and between analytical sessions. The method's accuracy is clear since the estimated relative standard deviations (RSD) are less than the maximum permissible value of 2%, designated as % RSDmax, as specified in Pharmacopoeias.

TABLE I. Intra and inter-day precision data

	Intraday		Interday	
nominal conc. (µg mL^{-1})	mean of observed peak area ± SD	% RSD	mean of observed peak area ± SD	% RSD
	Clobetasol propionate			
5	2574 ± 219.6	0.8532	26468 ± 477.2	1.8029
	Miconazole nitrate			
30	41433.7 ± 509.9	1.2308	41749 ± 475.7	1.1395
	Salicylic acid			
15	360081 ± 5978.6	1.6603	361016 ± 5840.2	1.6177

Accuracy

The accuracy was determined by calculating the percentage recovery of the observed concentration to the predicted concentration. To test the method's accuracy, recovery values were calculated for solutions containing 80%, 100%, and 120% of the specified clobetasol propionate, miconazole nitrate, and salicylic acid concentrations. Table II illustrates the findings. The procedure is amazingly precise, ensuring that dependable results are achieved.

TABLE II. Accuracy data for ointment

Level	Sample (µg mL^{-1})	Standard (µg mL^{-1})	Nominal conc. (µg mL^{-1})	Peak area	Recovery (µg mL^{-1})	% recovery
			Clobetasol propionate			
80	1	3	4	20130	3.99	99.75
100	1	4	5	23890	4.98	99.6
120	1	5	6	27420	5.91	98.5
			Miconazole nitrate			
80	20	4	24	30519	23.96	99.83
100	20	10	30	37326	29.83	99.43
120	20	16	36	44389	35.91	99.75
			Salicylic acid			
80	6	6	12	271455	11.98	99.83
100	6	9	15	325594	14.97	99.8
120	6	12	18	373902	17.92	99.55

Assay

A reversed-phase high-performance liquid chromatographic technique for simultaneous determination of clobetasol propionate, miconazole nitrate, and salicylic acid concentration in an ointment has been developed. The active components were monitored by measuring the peak area of the ointment and the standard, and the peak area ratio when calculated showed great recovery rates and assay %. The specificity of the chromatographic technique was established by screening a placebo solution and an assay solution together.

The placebo solution was made in the same way as the examined solution but without the drugs. (Table SVI, Supp. material), (Fig 2, 3. shows assay and placebo chromatogram for sample and standard)

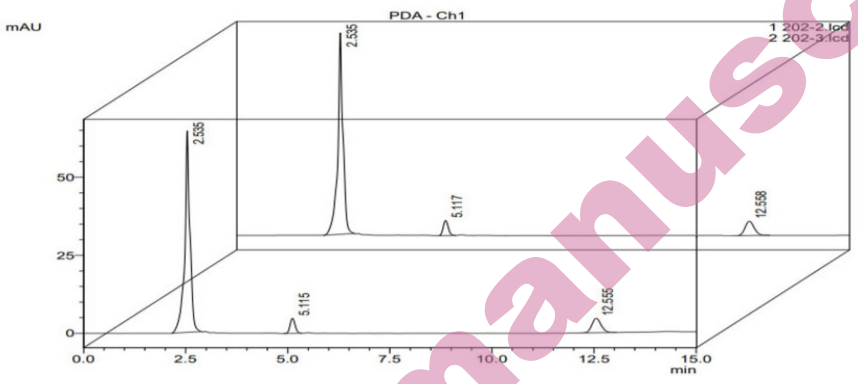

Fig 2. Assay chromatogram for sample and standard (concentration of the combined standard and sample solution is 100 µg mL^{-1})

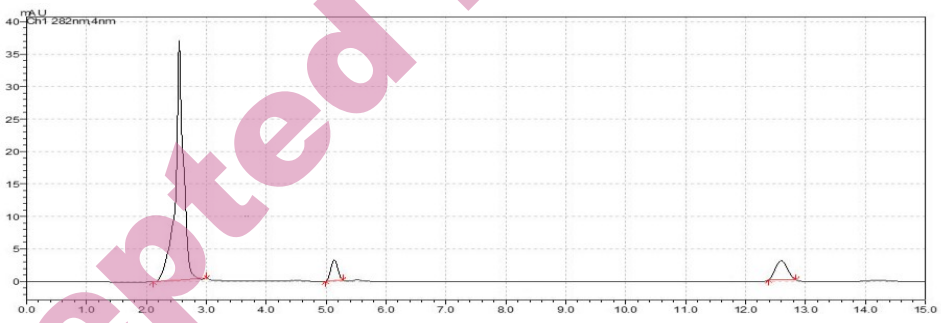

Fig 3. Specificity chromatogram contains 50 µg mL^{-1} of combined standard solution

Limit of detection and quantification

The detection and quantitation limits were calculated as LOD (k = 3.3) and LOQ (k = 10) and found to be 1.49 µg mL^{-1} and 4.53 µg mL^{-1} for clobetasol propionate, 8.72 µg mL^{-1} and 26.43 µg mL^{-1} for miconazole nitrate, and 3.37 µg mL^{-1} and 10.22 µg mL^{-1} for salicylic acid. (Table SVII, Supp. material)

Robustness

The robustness of the RP HPLC method was assessed by introducing minor, stochastic alterations to the wavelength, temperature, and flow rate. Variations were made to the flow rate (± 0.1 mL min^{-1}), temperature (± 2 °C), and wavelength (± 2 nm). As the determined relative standard deviations (RSD) are below the maximum acceptable value of 2% (RSDmax), as specified in Pharmacopoeias, the method's robustness is confirmed. (Table SVIII, SIX, SX, Supp. material)

CONCLUSION

The newly developed RP–HPLC and UV spectroscopy method using the formulated formulation provides a simple and precise method for simultaneously determining clobetasol propionate, miconazole nitrate, and salicylic acid in an updated ointment. This approach was distinguished by its simplicity, rapidity, and effectiveness. Validation data provided excellent precision and accuracy, confirming the suggested method's reliability.

SUPP. MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12827, or from the corresponding author on request.

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И З В О Д

РАЗВОЈ АНАЛИТИЧКЕ МЕТОДЕ И ВАЛИДАЦИЈА АНТИФУНГАЛНИХ ЛЕКОВА У АЖУРИРАНОЈ ФОРМУЛАЦИЈИ МАСТИ ПРИМЕНОМ UV СПЕКТРОСКОПИЈЕ И RP-HPLC

RETHINA KARUPPIAHYA[1], SUBA GEETHA ARUNACHALAM[1], SARAVANAN VENKATTAPURAM SAMPATH[1], SAMBATHKUMAR RAMANATHAN[1], ANANDA THANGADURAI SUBRAMANIAM[2], RAVIKUMAR RAMASAMY[1], ANUPRINCY PAULMURUGAN[1], JAMBULINGAM MUNUSAMY[1]

[1]*Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, Tamil Nadu, India,* [2]*Department of Pharmaceutical Analysis, JKKN College of Pharmacy, Kumarapalayam, Namakkal District, Tamil Nadu, India.*

Прецизна, једноставна и валидирана метода реверсно-фазне течне хроматографије високих перформанси (RP-HPLC) заједно са спектрофотометријском анализом је успостављена за истовремену квантификацију клобетасол-пропионата, миконазол-нитрата и салицилне киселине у ажурираној формулацији масти. Извршена је процена утицаја органских модификатора на задржавање циљних једињења. Хроматографска анализа је урађена коришћењем једноставне методе градијента ниског притиска са UV детекцијом на 282 nm на C18 колони (5 μm, 4,6 x 250 mm HSS). Мобилна фаза се састојала од смеше метанола, ацетонитрила и тетраетиламин ацетатног пуфера (pH 4) подешеног сирћетном киселином у односу 40:40:20 в/в, при брзини протока од 1,2 μg mL^{-1} . Метода је показала линеарност у опсегу концентрација од 5-15 μg mL^{-1} за клобетасол-пропионат, 30-90 μg mL^{-1} за миконазол-нитрат и 15-45 μg mL^{-1} за салицилну киселину. Одређене су границе детекције и квантификације: 1,49 μg mL^{-1} и 4,53 μg mL^{-1} за клобетасол-пропионат, 8,72 μg mL^{-1} и 26,43 μg mL^{-1} за миконазол-нитрат и 3,37 μg mL^{-1} и 3,37 μg mL^{-1} за салицилну киселину. Добијени одговор за лекове у формулацији је у опсегу од 95% до 99%.

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