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

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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 mm×250 mm HSS). The mobile phase consisted of a mixture of methanol, acetonitrile and tetraethylamine acetate buffer at pH 4, adjusted with acetic acid in a 40:40:20 volume 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 and 4.53 µg mL^{-1} for clobetasol propionate, 8.72 and 26.43 µg mL^{-1} for miconazole nitrate and 3.37 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 present 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

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calls for efficient treatment. If neglected, these infections can progress to more serious health issues, underscoring the importance of prompt intervention.[1]

Clobetasol propionate (Fig. S-2, of the Supplementary material to this paper) chemically designated as [(8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*S*,17*R*)-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 analogue of prednisolone, and it 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. S-3, Supplementary 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 inhibits the synthesis of ergosterol, a crucial component of fungal cell membranes, effective in the treatment of many fungal infections.[7,8]

Salicylic acid (Fig. S-1, Supplementary 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 analyse 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 the skin cell

turnover and the absorption of active ingredients. Using a polyethylene glycol base ensures the optimal penetration and the 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 the 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 the 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 min.

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 mm×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

About 15 g of PEG 4000 and 31 mL of PEG 400, was stirred until the mixture had achieved a semisolid consistency at room temperature. Then the fusion technique was used to add the appropriate quantities of clobetasol propionate, miconazole nitrate and salicylic acid in succession, ensuring thorough incorporation and a uniform distribution[23] (Table S-I, Supplementary material).

Preparation of standards and sample stock solution (RP-HPLC and UV–Vis 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, the aliquots of different concentrations were prepared by the 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 volume ratio).

A combined standard stock solution was prepared by accurately weighing and transferring 10 mg of clobetasol propionate, 60 mg of miconazole nitrate and 30 mg 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 and 300 μg mL^{-1} of salicylic acid. The final concentration was made to 50, 75, 100, 125 and 150 μg mL^{-1}, respectively by diluent. The final solutions were filtered using a membrane filter 0.45 μm of pore size.

The sample stock solution was prepared by accurately weighing 2 g of antifungal topical formulation which contains 0.01 g clobetasol propionate, 0.6 g of salicylic acid, 0.039 g of miconazole nitrate in a 100 mL volumetric flask, add 50 mL of ethanol and sonicate to dissolve. After lightly warming the solution to dissolve completely the volume was made with solvent ethanol for spectroscopic measurements (1000 μg 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 volume ratio).

Spectrophotometric analysis

Wavelength selection. From the standard stock, 20 μg mL^{-1} of the solution was prepared and scanned 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. S-4, Supplementary material).

MATLAB analysis

To achieve a concentration of 20 μg mL^{-1} for each analyte, dilutions were 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 Fig. 1), and MATLAB was employed to calculate the amount of drugs in the formulation.

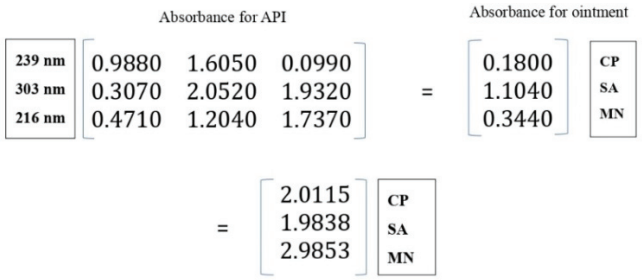

Fig. 1. A 3×3 matrix used for calculating drug content *via* MATLAB.

Chromatographic analysis

Linearity range. Typical linear concentrations were constructed with five samples of the 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.

The 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 the topical application. The 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 the skin application with 822,000 cps.

The spreadability of the new formulation was determined to be 17 cm^2 s^{-1}, which indicates the moderate to high spreadability rate. The 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 S-II, Supplementary 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 S-III, Supplmentary 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. The systematic experimentation revealed that a mobile phase containing methanol, acetonitrile and tetraethylamine acetate buffer at pH 4, adjusted with acetic acid in a 40:40:20 volume ratio, provided the optimal retention times for the target compounds, all in less than 15 min.

Given the solubility of all drugs in a methanol and acetonitrile mixture, for the initial trials these solvents were used. 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 pK_a 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 S-IV, Supplementary 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, 30–90 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 S-V and Fig. S-5, Supplementary material).

Precision

The triplicate samples of each drug were prepared and analysed using the proposed RP-HPLC technique on the same day and three days apart. The related coefficients of variation were then calculated. Table I 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 RSD_{max}, as specified in Pharmacopoeias.

Accuracy

The accuracy was determined by calculating the percentage recovery of the observed concentration related to the predicted concentration. To test the method's accuracy, the 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 I. Intra and inter-day precision data

Nominal conc. μg mL^{-1}	Intraday		Interday	
	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

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 the great recovery rates and the assay success percentage. 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 S-VI, Supplementary material). Figs. 2 and 3 shows the assay and the placebo chromatogram for sample and standard.

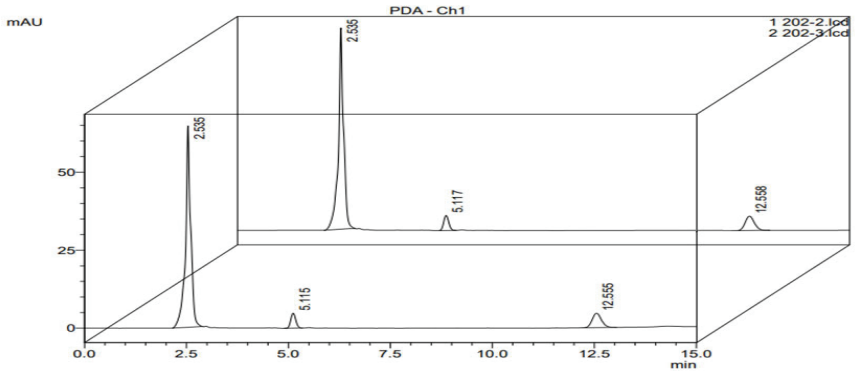

Fig. 2. Assay chromatogram for sample and standard (concentration of the combined standard and sample solution is 100 μg mL^{-1}).

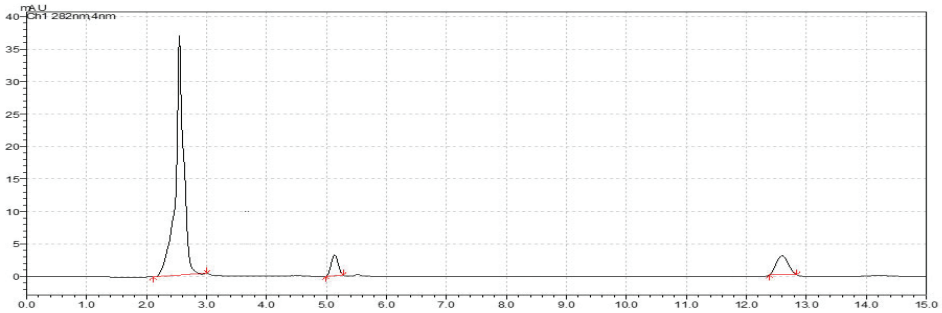

Fig. 3. Specificity chromatogram contains 50 μg mL^{-1} of combined standard solution.

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 and 4.53 μg mL^{-1} for clobetasol propionate, 8.72 and 26.43 μg mL^{-1} for miconazole nitrate and 3.37 and 10.22 μg mL^{-1} for salicylic acid (Table S-VII, Supplementary material).

Robustness

The robustness of the RP HPLC method was assessed by introducing minor, stochastic alterations to the wavelength, temperature and flow rate. The variations were made to the flow rate (±0.1 mL min^{-1}), the temperature (±2 °C) and the wavelength (±2 nm). As the determined relative standard deviations (*RSD*) are below the maximum acceptable value of 2 % (RSD_{max}), as specified in Pharmacopoeias, the method's reliability is confirmed (Tables SVIII–S-X, Supplementary material).

CONCLUSION

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

SUPPLEMENTARY MATERIAL

Additional data and information 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] и JAMBULINGAM MUNUSAMY[1]

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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 састава масти за кожу. Извршена је процена утицаја органских модификатора на синтезу 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 mm×250 mm HSS). Мобилна фаза се састојала од смеше метанола, ацетонитрила и тетраетиламин ацетатног пуфера (pH 4), подешеног сирћетном киселином у запреминском односу 40:40:20, 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 и 4,53 μg mL^{-1} за клобетасол-пропионат, 8,72 и 26,43 μg mL^{-1} за миконазол-нитрат и 3,37 и 3,37 μg mL^{-1} за салицилну киселину. Добијени резултат за лекове у формулацији је у опсегу од 95 до 99 %.

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