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Development and validation of different spectrophotometric and chromatographic methods for determination of clotrimazole and hydrocortisone in a topical cream

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ABSTRACT

In this study, we developed and validated different spectrophotometric and chromatographic methods for determination of clotrimazole (CLO) and hydrocortisone (HDC) in their combined dosage form. The developed spectrophotometric methods were first derivative spectrophotometry (^{1}D) by measuring the peak amplitude at 247.4 and 236.2 nm for CLO and HDC, respectively, second derivative of ratio spectra (^{2}DD) at 225.4 nm for CLO and 269 nm for HDC, dual wavelength spectrophotometry (DW) by measuring absorbance difference between 225.4 and 264 nm for CLO and between 228 and 247 nm for HDC determination, advanced absorbance subtraction method (AAS) between 225.4 and 264 nm and mean centering of ratio spectra (MCR) spectrophotometric method in the range of 232-265 nm. On the other hand, the proposed chromatographic method was Ultra Performance Liquid Chromatography method (UPLC) using acetonitrile:water (50:50, *v:v*) as a mobile phase and the peaks were detected at 228 nm. All these methods were successfully applied to determine the two studied drugs in pure forms; laboratory prepared mixtures and combined dosage form. Methods validations were carried out regarding linearity, accuracy, precision and selectivity. The spectrophotometric methods exhibited a linear dynamic range over 5-40 and 5-45 µg/mL for CLO and HDC, respectively, and over 3-35 and 5-50 µg/mL for UPLC method. Sensitive and selective spectrophotometric and UPLC methods for the determination of clotrimazole and hydrocortisone in their topical formulation were successfully developed and validated. The developed methods were statistically confirmed to be accurate, precise and reproducible.

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1. Introduction

Clotrimazole is chemically known as, 1-(o-chloro-α,α-di phenylbenzyl)imidazole [1], it is an imidazole derivative with a broad spectrum antimycotic activity. It inhibits biosynthesis of the sterol ergostol; an important component of fungal cell membranes. Its action leads to increase in membrane permeability and apparent disruption of enzyme systems bound to the membrane [2-4].

Hydrocortisone is chemically known as, 11,17,21-tri hydroxypregnan-4-ene-3,20-dione [1], it is a principal glucocorticoid hormone. It is produced by the adrenal cortex and has been used to treat skin problems such as rashes and eczema. It is also used to treat many immune and allergic disorders [2-4]. The combination of these drugs is indicated topically for the treatment of skin infections due to dermatophytes, moulds and other fungi.

Surveying the literature reveals various methods for determination of these drugs separately; for CLO; it was analyzed by titrimetric [1,5], spectrophotometric [6,7], TLC [8] and HPLC methods [9-11], for HDC; polarographic [12,13], spectrophotometric [1,14], fluorimetric [15], TLC [16], HPLC [17-22] and LC-MS/MS [23-25] methods. There is only one reported HPTLC method has been published for simultaneous analysis of CLO and HDC [26]. The aim of this work was to develop and validate novel different spectrophotometric and chromatographic methods for the determination of CLO and HDC in raw materials and in the topical cream. The suggested methods are simple, rapid, accurate, selective and less time consuming (as especially for UPLC of analysis time less than 3 min) compared with the published HPTLC method. The proposed methods have been optimized and validated as per ICH guidelines [27] and were found to obey the acceptance criteria.

2. Experimental

2.1. Instrumentation

A double beam UV-visible spectrophotometer (Shimadzu, Japan) model UV-1601 PC with quartz cell of 1 cm connected

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to an IBM compatible PC, an HP-600 inkjet printer, and UV-PC personal software version 3.7 were used. The spectral bandwidth used was 2 nm and wavelength-scanning speed was 2800 nm/min. Sonix TV SS-series ultrasonicator (USA) was used for extraction of drugs from their pharmaceutical formulation. PLS-Toolbox for use with MATLAB® 7.1 used for mean centering of ratio spectra method. Dionex Ultimate 3000 UPLC equipped with a Quaternary solvent delivery pump, auto-sampler and diode array detector (Germany) were used in UPLC method. The output signal was monitored and processed using Chromeleon software version 7.2.1.5537. Digital balance (Sartorius, German) and Jenway 3505 pH meter (Stafford shine, UK) were used.

2.2. Reagents and chemicals

All solvents were of HPLC grade and the chemicals used were of analytical grade; acetonitrile and methanol (Fisher, Loughborough, UK), hydrochloric acid, analytical grade (El-Nasr Pharmaceutical Chemical Co., Abu-Zabaal, Cairo, Egypt). De-ionized water (SEDICO pharmaceuticals Co., 6th October City, Egypt). Solvents and chemicals used for UPLC were filtered through 0.45 µm membrane filters (Sartorius Stedium Biotec. GmbH, Goettingen, Germany).

2.3. Standards

Standard CLO was kindly supplied by Alexandria Company for Pharmaceuticals and Chemical Industries (Alexandria, Egypt), while HDC was kindly donated by Kahira Pharmaceuticals and Chemical Industries Company (Cairo, Egypt). Their purity was found to be 98.70 and 99.25% for CLO and HDC, respectively, according to the reported method [26].

2.4. Samples

Dermatin Cort® cream (Batch No. 804) was manufactured by PHARCO, for pharmaceutical and chemical industry (Alexandria, Egypt), each gram of the cream claimed to contain 10 mg of CLO and 10 mg of HDC.

2.5. Preparation of standard solutions

2.5.1. For spectrophotometric methods

Standard stock solutions of CLO and HDC were prepared in methanol in the concentration of 1 mg/mL. Standard working solutions of CLO and HDC were prepared in 0.05 N HCl in the concentration of 0.1 mg/mL.

2.5.2. For UPLC method

Standard stock solutions of CLO and HDC were prepared in acetonitrile in the concentration of 1 mg/mL. Standard working solutions of CLO and HDC were prepared in the mobile phase (acetonitrile and de-ionized water, 50:50, *v*:*v*), respectively, in the concentration of 0.1 mg/mL.

2.6. Laboratory-prepared mixtures

Different mixtures containing different ratios of CLO and HDC were prepared from their respective working solutions (0.1 mg/mL) into series of 10 mL volumetric flasks and the volume was completed to the mark with 0.05 N HCl for spectrophotometric methods.

2.7. Pharmaceutical formulation

The content of three Dermatin Cort® cream tubes was mixed well. One g of the cream containing 10 mg each of CLO and HDC was taken into a 200 mL beaker. Methanol (30 mL) was added and the cream was allowed to melt by warming at 70 °C in a hot water in ultrasonicator. The melted cream was left to solidify at room temperature. The warming and cooling process was repeated for three times. The liquid portion was transferred to a 100 mL volumetric flask. Extract process were repeated three times (15 mL ×3), warming and cooling process were repeated while liquid portions were collected in the volumetric flask. The volume was completed with the proper solvent to obtain working solution of 0.1 mg/mL of either CLO or HDC. Then samples within linearity ranges were prepared. Standard addition technique had been also carried out.

2.8. Procedures

2.8.1. For ultra-performance liquid chromatography method

2.8.1.1. Chromatographic conditions

Chromatographic separation was performed on a Hypersil Gold C18 column (150 mm × 4.6 id, 3 µm particle size) and the mobile phase was prepared by mixing acetonitrile: water in the ratio of 50:50 (*v*:*v*). The mobile phase was filtered through 0.45 µm Millipore membrane filters and degassed for 10 min using ultrasonicator prior to use. Injection volume was 20 µL; flow rate was maintained at 1 mL/min under pressure of 90 bars. UV detection of the effluent was performed at 228 nm.

2.8.1.2. Construction of calibration curves

Aliquots equivalent to 3-35 and 5-50 µg/mL of CLO and HDC, respectively, were prepared from their respective working solutions (0.1 mg/mL) into two separate series of 10 mL volumetric flasks, then the volume was completed with acetonitrile: water (50:50, *v*:*v*). 20 µL of each concentration was injected in triplicates. The relative peak area (obtained by dividing the integrated peak areas of each drug by the area of 25 µg/mL of its corresponding solution as an external standard) was used to construct calibration curve of each component from which its regression equation was computed.

2.8.2. For spectrophotometric methods

2.8.2.1. Spectral characteristics of CLO and HDC

Zero order absorption spectra of 20 µg/mL each of CLO, HDC and their laboratory mixture containing equal concentrations of both drugs (each 10 µg/mL) were recorded in the UV range of 200-400 nm using 0.05 N HCl as a solvent.

2.8.2.2. Construction of calibration curves

Accurately measured aliquots equivalent to 50-400 and 50-450 µg of CLO and HDC, respectively, were transferred from their working solutions (0.1 mg/mL) into two series of 10 mL volumetric flasks. The volume was completed with 0.05 N HCl.

2.8.2.3. First derivative spectrophotometric method

^{1}D curves were recorded using Δλ = 4 nm and scaling factor = 10. The peak amplitudes at 247.4 and 236.2 nm for CLO and HDC, respectively, were recorded, and then calibretion graphs were constructed. The regression equations were then computed for the studied drugs.

2.8.2.4. Second derivative of ratio spectra spectrophotometric method

^{2}DD spectra of CLO were obtained using 10 µg/mL of HDC as a divisor, Δλ = 8 nm and scaling factor = 10 while the ^{2}DD

spectra of HDC were obtained using standard spectrum of 10 µg/mL CLO as a divisor, Δλ = 8 nm and scaling factor = 10. ^{2}DD peak amplitudes were measured at 225.4 and 269 nm CLO and HDC, respectively. The calibration curves were constructed relating the peak amplitudes against the corresponding concentrations and the regression equations were calculated.

2.8.2.5. Dual wavelength method

For determination of CLO, a calibration curve was plotted relating the absorbance difference between 225.4 and 264 nm against its corresponding concentrations and the regression equation was computed. Similarly, HDC was determined using the absorbance difference between 228 and 247 nm and the corresponding regression equation was calculated.

2.8.2.6. Advanced absorbance subtraction method

The absorbance of scanned spectra of CLO in the range of 5-40 µg/mL was measured at 225.4 (isoabsorptive point, λ_{iso}) and 264 nm. Calibration graph was constructed relating its absorbance at the isoabsorptive point (225.4 nm) versus the corresponding concentrations of CLO and the regression equation was formulated. Additionally, a line representing the relation between the absorbance difference at 225.4 and 264 nm against the absorbance at isoabsorptive point (225.4 nm) was plotted, and then the regression equation was formulated.

2.8.2.7. Mean centering of ratio spectra method

The absorption spectra of the prepared solutions of CLO in the concentration range of 5-40 µg/mL were recorded in the range of 232-265 nm then divided by the standard spectrum of 20 µg/mL of HDC the obtained ratio spectra were then mean centered. By the same way the absorption spectra of HDC in the concentration range of 5-45 µg/mL were recorded in the range of 232-265 nm, were divided by the standard spectrum of 20 µg/mL of CLO and then the obtained ratio spectra were mean centered. Calibration curves of the developed drugs were constructed by plotting amplitude values between 236 and 254 nm (peak to peak) for CLO and the amplitude values between 247 and 264 nm (peak to peak) for HDC against their corresponding concentrations.

2.8.2.8. Analysis of laboratory prepared mixtures

The procedure given under construction of calibration curves has been followed using the previously prepared mixtures. Concentrations of CLO and HDC were then calculated by the aid of the corresponding computed regression equations.

2.9. Application of the methods to pharmaceutical formulation

The procedure under construction of calibration graphs was followed using the previously prepared samples of Dermatin Cort® cream. Concentrations of CLO and HDC were calculated from their respective regression equations and the percentage recoveries were then calculated.

3. Results and discussion

Clotrimazole and hydrocortisone act as antimycotic drugs which indicated for the treatment of many skin infections and co-existing symptoms of inflammation. So, it is very important to develop new analytical methods which are accurate, precise and rapid for determination of studied drugs in their pharmaceutical formulation without preliminary separation. The main problem in the spectrophotometric analysis of CLO and HDC binary mixture was the spectral overlap of the two proposed drugs which hindered their direct determination. To overcome this problem, we established different spectrophotometric methods which are money and time saving, in addition to the more rapid chromatographic one (UPLC) which is characterized by its short analysis time, high selectivity and precision.

3.1. Methods optimization

Methods were optimized to develop quantitative methods with those offer high resolution between the drugs in a short time of analysis. It was necessary to check the effect of different parameters affecting methods selectivity, sensitivity and efficiency.

3.1.1. For spectrophotometric methods

Parameters affecting resolution between CLO and HDC were studied and optimized. These parameters are solvent and divisor concentration.

3.1.1.1. Solvent

Different solvents were tested to choose the suitable one (Methanol, ethanol, de-ionized water, 0.05 N HCl and 0.05 N NaOH). It was found that 0.05 N HCl was the best solvent.

3.1.1.2. Divisor concentration

The analytical parameters such as intercept, slope and the correlation coefficient of calibration equation are affected by the divisor concentration, so it was tested. Different concentrations of CLO and HDC were tested as divisors (10, 15, 25 and 30 µg/mL). It was found that 10 µg/mL was the best concentration for a ^{2}DD method and 20 µg/mL was the most suitable one for MCR method.

3.1.2. For UPLC method

3.1.2.1. Mobile phase

Different mobile phases with different compositions and ratios were tested to get the best chromatographic separation. First trial began with water: methanol in different ratios. Asymmetric tailed and forked peaks have been obtained. The second trial was to test the effect of addition of different ratios of either acetic acid or triethylamine, but no improvement in separation was observed. The last trial was to replace methanol with acetonitrile that led to improving in the shape of the peaks and hence the resolution between them. Different ratios of water: acetonitrile were tried. Finally, the best separation was achieved by using a mobile phase consisted of acetonitrile: water (50:50, *v*:*v*) with a flow rate of 1 mL/min on a C18 column.

3.1.2.2. Scanning wavelength

Different scanning wavelengths were tested like (205, 210, 228 and 254 nm) to choose the most sensitive wavelength. The wavelength 228 nm gave a good sensitivity for all the components. Finally, good resolution and acceptable peak shape have resulted where the tree of HDC was 1.853 and of CLO was 2.087, as in Figure 1.

3.2. Application of the methods

3.2.1. First derivative spectrophotometric method

The zero order absorption spectra of CLO and HDC displayed severe overlapping, as shown in Figure 2.

Table 1. Regression and analytical parameters of the proposed methods for the determination of clotrimazole and hydrocortisone.

Methods	^{1}D		^{2}DD		DW		AAS		MCR		UPLC	
Parameters	CLO	HDC	CLO	HDC	CLO	HDC	CLO	HDC	CLO	HDC	CLO	HDC
Calibration range (µg/mL)	5-40	5-45	5-40	5-45	5-40	5-45	5-40	5-45	5-40	5-45	3-35	5-50
Slope	0.0057	0.0131	0.0221	0.0301	0.0106	0.0179	0.0190	0.0192	0.0081	0.0427	0.0366	0.0376
Intercept	0.0022	0.0047	0.0009	0.1454	0.0129	0.0419	0.0344	0.0052	0.0127	0.2139	0.0881	0.0509
Correlation coefficient (*r*)	0.9999	0.9999	0.9999	0.9999	0.9999	0.9998	0.9999	0.9999	0.9999	0.9999	0.9998	0.9999
Accuracy, Mean ±RSD%	99.21 ±0.670	99.87 ±1.070	99.95 ±0.560	100.17 ±1.060	99.68 ±0.910	99.60 ±0.840	99.79 ±0.610	100.12 ±0.720	99.75 ±0.750	100.08 ±0.830	99.95 ±1.170	100.13 ±0.760
Repeatability (RSD%) [1,2]	0.005	0.014	0.006	0.004	0.016	0.014	0.013	0.007	0.008	0.001	0.003	0.001
Intermediate Precision (RSD%) [2]	0.007	0.018	0.015	0.009	0.090	0.020	0.015	0.012	0.011	0.006	0.821	0.003
LOD (µg/mL) [3]	1.21	1.26	1.13	1.38	1.56	1.14	1.39	1.44	1.43	1.18	0.70	1.04
LOQ (µg/mL) [4]	3.65	3.81	3.44	4.19	4.72	3.46	4.21	4.37	4.32	3.58	2.13	3.14

[1] (RSD%) of 3 concentrations (10, 15, 25 µg/mL) of each component (CLO and HDC) on the same day.
[2] (RSD%) of 3 concentrations (10, 15, 25 µg/mL) of each component (CLO and HDC) on three successive days.
[3] LOD = 3.3*SD/Slope.
[4] LOQ = 10*SD/Slope.

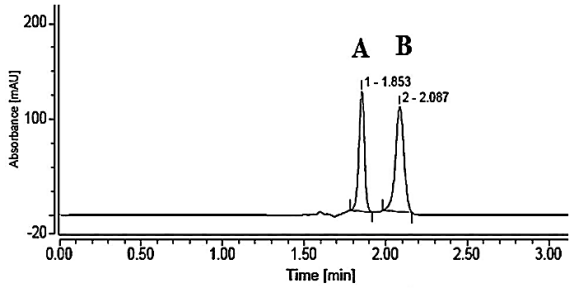

Figure 1. UPLC chromatogram of a mixture of (A) HDC and (B) CLO using acetonitrile: water (50:50, *v:v*) as a mobile phase with UV detection at 228 nm.

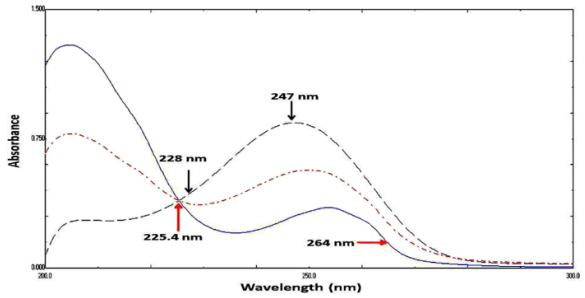

Figure 2. Zero order absorption spectra of 20 µg/mL each CLO (—), HDC (---) and the binary mixture of CLO and HDC containing 10 µg/mL each (—··—) using 0.05 N HCl as a solvent.

This overlapping restricted the direct determination of studied drugs. Derivative spectrophotometry is an analytical method of great utility for resolving the overlapped spectra [28]. The application of ^{1}D method provided a selective determination of CLO and HDC by measuring the peak amplitude at 247.4 and 236.2 nm, respectively, as shown in Figure 3. The calibration curves were constructed and the regression equations were given in Table 1.

3.2.2. Second derivative of ratio spectra spectrophotometric method

Another method for resolving binary mixtures without prior separation is the second derivative of the ratio spectrum spectrophotometric method (^{2}DD) [29]. The main advantage of the method is that the whole spectrum of an interfering substance is canceled. Accordingly, the choice of the selected is not critical as in the derivative spectrophotometric methods. Dividing the absorption spectra of HDC by the standard spectrum of 10 µg/mL of the CLO as a divisor; the obtained ratio spectra was subjected to the second derivatization using Δλ = 8 nm and scaling factor = 10 as shown in Figure 4. On the other hands the absorption spectra of CLO were divided by 10 µg/mL of HDC as a divisor, then the second derivative for the obtained spectra was recorded using Δλ = 8 nm and scaling factor = 10, Figure 5. The peak amplitudes of the second derivative of ratio spectra at 225.4 and 269 nm were plotted versus the concentrations of CLO and HDC, respectively. ^{2}DD values showed good linearity and reproducibility at these wavelengths, regression parameters were presented in Table 1.

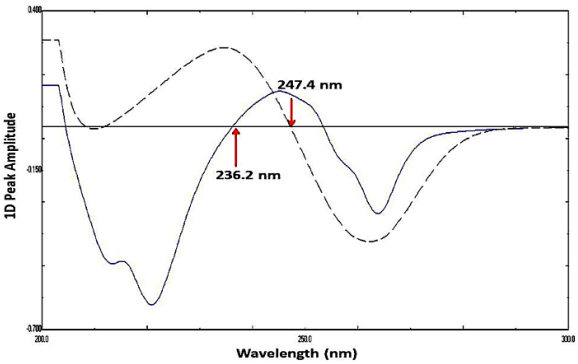

Figure 3. First derivative absorption spectra of 20 µg/mL each of CLO (—) & HDC (---) using 0.05 N HCl as a solvent.

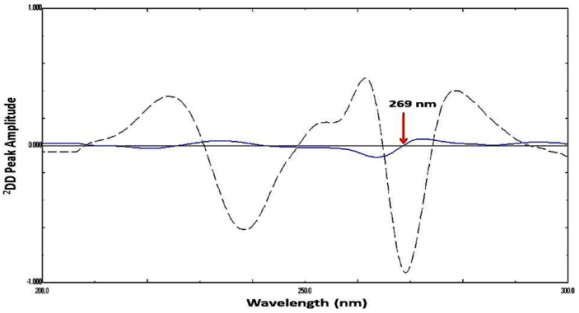

Figure 4. Second derivative of ratio spectra of 20 µg/mL each CLO (—) and 20 µg/mL of HDC (---) using 10 µg/mL of CLO as a divisor and 0.05 N HCl as a solvent.

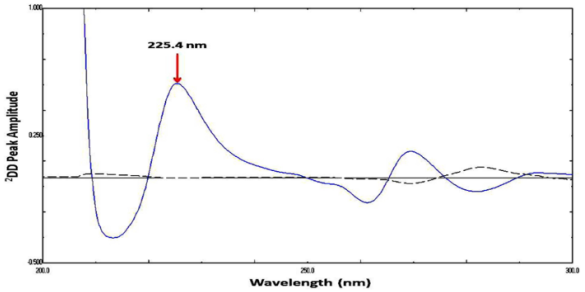

Figure 5. Second derivative of ratio spectra of 20 µg/mL each of CLO (—) and HDC (---) using 10 µg/mL of HDC as divisor and 0.05 N HCl as a solvent.

3.2.3. Dual wavelength analysis method

Different wavelengths were tried to develop a dual-wavelength method, the best results regarding sensitivity and selectivity were obtained by using the absorbance difference between 225.4 and 264 nm for CLO (where HDC has the same absorbance), 228 and 247 nm for HDC (where CLO has zero difference) as shown in Figure 2. Calibration graphs for both drugs were constructed by plotting the difference in absorbance values at the chosen wavelengths for each drug against their corresponding concentrations. CLO and HDC obeyed Beer Lambert's law in the concentration ranges of 5-40 and 5–45 µg/mL for CLO and HDC, respectively, with good correlation coefficients as in Table 1.

3.2.4. Advanced absorbance subtraction method

The absorption spectra of the standard solutions of CLO with different concentrations were recorded in the wavelength range of 200-400 nm. Two wavelengths were selected 225.4 and 264 nm where HDC showed equal absorbance at the selected wavelengths Figure 2. So the absorbance difference ΔA (A_{iso}-A_{264}) between the two selected wavelengths on the mixture spectra is directly proportional to the concentration of CLO; [30,31]. A calibration graph was plotted for CLO representing the relationship between the absorbance difference (A_{iso}-A_{264}) against A_{iso} and the following regression equation was formulated:

$$\Delta A\ (A_{iso}\text{-}A_{264}) = 0.5115\ A_{iso} + 0.003 \quad r = 0.9999 \qquad (1)$$

where ΔA is the absorbance difference between two selected wavelengths for CLO spectra, A_{iso} is the absorbance at 225.4 (λ_{iso}) of CLO. By substituting using the absorbance difference ΔA (A_{iso}-A_{264}) between the two selected wavelengths of the mixture spectrum in the previous equation, the postulated absorbance A corresponding to CLO at A_{iso} was obtained. Subtracting the obtained absorbance of CLO at A_{iso} from the practically recorded absorbance [$A_{Recorded}$] at A_{iso} to get that corresponding to HDC as:

$$A_{Recorded} = A_{HDC} + A_{CLO} \qquad (2)$$

By plotting the absorbance of zero order spectra of CLO or HDC at A_{iso} 225.4 nm against the corresponding concentrations, a unified regression equation was calculated and was used for determination of both CLO and HDC using the calculated absorbance value of each at λ_{iso}.

3.2.5. Mean centering of ratio spectra method

The developed MCR method is based on the mean centering of ratio spectra; it gets rid of the derivatization steps and therefore signal-to-noise ratio is increased. A mathematical explanation of the developed method was illustrated by Afkhami and Bahram [32]. Since the wavelength range taken has great effect on the obtained mean centering ratio spectra, different wavelength ranges were tested and the best results were given when using the wavelength range from (232-265 nm) for both CLO and HDC. Also, the divisor concentration was selected with care to obtain maximum selectivity and sensitivity and the best results were obtained on using 20 µg/mL each of studied drugs as divisors. The absorption spectra of the standard solutions of CLO with different concentrations were recorded and divided by the standard spectrum of 20 µg/mL of HDC then mean centering of the resulted ratio spectra has been obtained, Figure 6. By the same way, standard solutions of HDC with different concentrations were recorded and divided by 20 µg/mL CLO. Then mean centering of the resulted ratio spectra has been obtained, Figure 7. The amplitudes of the obtained mean centered ratio spectra at 236-254 nm (peak to peak) and at 247-264 nm (peak to peak) was recorded for both CLO and HDC, respectively. Calibration graphs were plotted and the regression equations were calculated and given in Table 1.

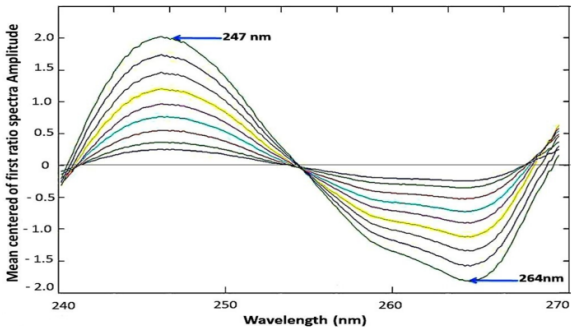

Figure 6. Mean centered of first ratio spectra of CLO (5-40 µg/mL) using 20 µg/mL of HDC as a divisor and 0.05 N HCl as solvent.

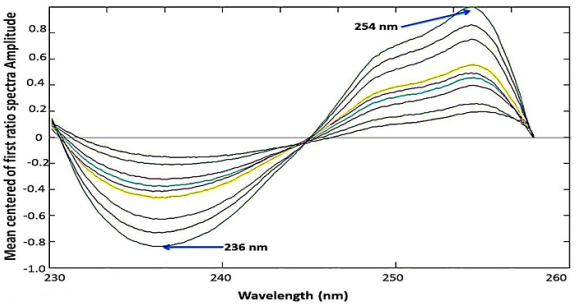

Figure 7. Mean centered of first ratio spectra of HDC (5-45 µg/mL) using 20 µg/mL of CLO as a divisor and 0.05 N HCl as solvent.

3.2.6. Ultra-performance liquid chromatography method

Separation was carried out at ambient room temperature using Hypersil Gold C18 column (150 mm × 4.6 id's, 3 µm particle sizes) using a mixture of acetonitrile: water in the ratio of (50:50, *v:v*) as a mobile phase with flow rate 1 mL/min under pressure of 90 bars and UV detection at 228 nm. The studied drugs had been separated where the t_R of HDC = 1.853 and of CLO = 2.087 as shown in Figure 1.

3.3. Method validation

Methods validation was performed with respect to ICH guidelines [27] like linearity, accuracy, precision, LOD and LOQ were measured and the results are given in Table 1.

Table 2. Determination of CLO and HDC in laboratory-prepared mixtures by the proposed spectrophotometric methods.

Mix No	Conc. of CLO: HDC	Ratio of CLO: HDC	% Recovery									
			[1]D		[2]DD		DW		AAS		MCR	
			CLO	HDC	CLO	HDC	CLO	HDC	CLO	HDC	CLO	HDC
1	10:10	1:1	100.35	101.76	99.24	99.53	101.98	99.50	100.32	101.46	100.86	100.68
2	20:10	2:1	98.42	97.94	98.21	99.20	99.10	99.50	102.08	100.42	101.05	100.21
3	20:20	1:1	98.42	99.35	98.44	100.76	100.05	99.19	98.47	99.79	101.05	101.74
4	10:30	1:3	98.60	100.84	100.05	99.07	101.98	100.76	98.17	101.53	100.86	101.79
5	10:25	2:5	98.60	101.77	100.95	99.75	99.15	101.03	98.21	99.42	100.86	102.47
6	25:15	5:3	99.79	99.90	101.19	100.69	101.55	99.85	99.77	98.89	98.62	101.31
Mean±RSD%			99.03 ±0.83	100.26 ±1.50	99.68 ±0.73	99.83 ±0.73	100.64 ±0.75	99.97 ±0.75	99.50 ±1.54	100.25 ±1.08	100.55 ±0.95	101.37 ±0.82

Table 3. Quantitative determination of CLO and HDC in Dermatin Cort® cream by the proposed methods and application of standard addition technique.

Drugs		Parameters	Methods					
			[1]D	[2]DD	DW	AAS	MCR	UPLC
Dermatin Cort ® cream (batch No. 804)	Clotrimazole	Taken (µg/mL)	10.00	10.00	10.00	10.00	10.00	10.00
		Found %±RSD% [1]	99.18±1.43	97.03±0.98	96.79±1.30	96.98±1.87	98.85±1.75	97.24±1.39
		Pure added (µg/mL)	8.00	8.00	8.00	8.00	8.00	8.00
			10.00	10.00	10.00	10.00	10.00	10.00
			15.00	15.00	15.00	15.00	15.00	12.00
		% Recovery	98.69	100.11	100.23	100.77	100.31	99.28
			100.00	100.45	100.94	99.56	98.77	100.86
			100.59	100.15	99.37	99.36	99.59	100.63
		Mean±RSD%	99.76±0.97	100.24±0.19	100.18±0.79	99.90±0.77	99.56±0.77	100.26±0.85
	Hydrocortisone	Taken (µg/mL)	10.00	10.00	10.00	10.00	10.00	10.00
		Found %±RSD% [1]	97.18±1.37	99.09±1.20	97.45±1.26	98.25±1.85	98.34±1.84	99.64±0.88
		Pure added (µg/mL)	8.00	8.00	8.00	8.00	8.00	8.00
			10.00	10.00	10.00	10.00	10.00	10.00
			15.00	15.00	15.00	15.00	15.00	12.00
		% Recovery	100.75	100.09	99.97	100.72	99.53	100.25
			99.69	100.00	100.09	99.85	100.94	99.20
			100.05	100.78	99.87	100.24	100.70	99.17
		Mean±RSD%	100.16±0.54	100.29±0.43	99.98±0.11	100.27±0.44	100.39±0.75	99.65±0.62

[1] All data are represented as mean±SD; n = 6.

Table 4. Parameters of system suitability testing of the developed UPLC method for the determination of CLO and HDC.

Parameters	HDC	CLO	Reference values [33]
Symmetry factor	0.94	0.93	For a typical 1 symmetric peak
Resolution (Rs)	3.08		> 2
Selectivity (α)	1.92		> 1.5
Column efficiency (N)	14911	8395	Increases with efficiency of the separation
HETP (cm/plate) [1]	0.001	0.002	The smaller the value, the higher the column efficiency

[1] HETP: Height equivalent to theoretical plates.

3.3.1. Specificity

The specificity of these methods was achieved by analysis of different synthetic prepared mixtures of CLO and HDC within their linearity ranges. Good results were obtained from all the proposed methods shown in Table 2. These results confirmed that both CLO and HDC did not interfere with each other. By applying the developed methods to Dermatin Cort ® cream good percentage recoveries were obtained which proved that excipients existed in cream did not interfere with any of separated drugs and ensuring its selectivity, Table 3.

3.3.2. Robustness

Robustness of the developed UPLC method was proved when we tried to make minor deliberate changes in some parameters like changing in % acetonitrile in the mobile phase ±2%, changing in scanning wavelength ±2 nm and changing at flow rate ±0.05 mL/min. By calculating the % RSD of peak areas for each of the studied components after each trial, it was found to be 0.009 and 0.008 for CLO and HDC, respectively in changing % of mobile phase ±2%, 0.012 and 0.018 for CLO and HDC, respectively in changing the flow rate ±0.05 mL/min. They have been confirmed that changing in the studied parameters did not have a significant or dramatic effect on the peak areas.

3.3.3. System suitability

Parameters including resolution (Rs) and selectivity (α) factors were calculated for both CLO and HDC and were found to be > 2 and 1.5, respectively. In addition, the peak symmetry factors were calculated for the two components and nearly equal 1 [33]. Other parameters such as height equivalent to theoretical plates and the number of theoretical plates were calculated, and their values were within the acceptable limits, Table 4.

3.3.4. Statistical analysis

The results of the developed methods for determination of CLO and HDC were statistically compared with those obtained by applying a reported HPTLC method [26] for Dermatin Cort ® cream using Student's *t*-test and variance ratio *F*-test at 95% confidence level. The calculated *t* and *F* values were less than the reported ones, confirming that there is no significant difference between them with respect to accuracy and precision, Table 5.

4. Conclusions

The developed methods have been successfully applied for the determination of CLO and HDC in pure and in their combined formulation, they were found to be simple, rapid and accurate methods. The proposed UPLC method had some advantages of being highly selective, sensitive and has short analysis time (less than 3 min), hence lower solvent consumption over other HPLC methods. Also, the proposed spectrophotometric methods were found to be rapid, simple, accurate and less expensive. The suggested methods were completely validated and satisfactory results were obtained.

Table 5. Statistical comparison of the results obtained by the proposed methods and the reported HPTLC method for the analysis of Dermatin Cort® cream.

Methods	**[1]D**		**[2]DD**		**DW**		**AAS**		**MCR**		**UPLC**		**Reported Method [1] [26]**	
Parameters [2]	**CLO**	**HDC**	**CLO**	**HDC**	**CLO**	**HDC**	**CLO**	**HDC**	**CLO**	**HDC**	**CLO**	**HDC**	**CLO**	**HDC**
Mean (%)	98.96	97.18	97.03	99.09	96.79	97.45	96.98	98.25	98.85	98.34	97.24	99.64	98.70	98.25
S.D	1.67	1.37	0.99	1.30	1.30	1.26	1.87	1.85	1.75	1.84	1.39	1.88	0.98	1.24
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Student's t-test (2.228) [3]	1.870	1.008	0.876	0.905	1.184	1.304	1.345	1.635	1.258	1.709	1.374	1.250		
F-test (5.505)	2.905	1.220	1.021	1.099	1.760	1.032	3.643	2.225	3.190	2.201	2.013	2.298		

[1] Reported HPTLC method for determination of CLO and HDC using Toluene: Propanol: Ammonia (13:3:0.1, *v*:*v*:*v*) as developing system reflectance/absorbance mode at 226 nm.
[2] All data are represented as mean±SD; n=6.
[3] Figures between parentheses represent the corresponding tabulated values of t and F (p = 0.05).

Recovery studies indicated that there was no interference from cream additives, the developed methods can be easily applied to quality control of the studied drugs. Moreover, the obtained results confirmed the accuracy and precision of these methods.

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