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Different spectrophotometric methods manipulating ratio spectra for the assay of hydrocortisone acetate and clioquinol in their topical preparation

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ABSTRACT

Simple and precise spectrophotometric methods for quantitative assay of a mixture of hydrocortisone acetate (HCA) and clioquinol (CL) were developed and validated through different mathematical manipulation pathways. The developed methods utilized ratio spectra for resolving binary mixtures including absorbance subtraction, ratio subtraction coupled with spectrum subtraction, constant multiplication, constant value, and derivative ratio. The proposed methods were proved to be specific by analysing the laboratory-prepared mixtures and were applied for the assay of topical preparation successfully. The methods were validated using ICH guidelines where accuracy, repeatability and intermediate precision were within the acceptable limits. The linearity range was found to be 2-22 for HCA and 1.5-7 µg/mL for CL in all proposed methods and 2-7 µg/mL for HCA and CL in absorbance subtraction method through using a unified regression equation. The findings were statistically evaluated with respect to the official and reported methods, demonstrating that there was no significant difference.

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1. Introduction

Hydrocortisone acetate, [2-[(8S,9S,10R,11S,13S,14S,17R)-11, 17-dihydroxy-10, 13-dimethyl-3-oxo-2, 6, 7, 8, 9, 11, 12, 14, 15, 16-decahydro-1H-cyclopenta[a]phenanthren-17-yl]-2-oxoethyl]acetate, (Figure 1a), is a principal glucocorticoid hormone [1-3]. It is produced by the adrenal cortex and has been used clinically to treat skin problems such as rashes, eczema and others. Clioquinol, 5-chloro-7-iodoquinolin-8-ol (Figure 1b), is a halogenated hydroxyquinoline with antibacterial and antifungal activity [1]. The combination of the anti-inflammatory action of HCA with the antibacterial action of CL has been proved to be highly effective in skin disorders [4].

Hydrocortisone, clioquinol and their binary dosage form are official in British Pharmacopoeia [5] and USP [6]. Several methods for the determination of the drugs have been reported in different dosage forms [7-13]. Literature survey revealed that HCA and CL binary mixture was determined by HPLC and TLC methods [14,15]. No spectrophotometric methods have been developed for the determination of both drugs simultaneously in different mixtures.

Studying different methods manipulating ratio spectra in the analysis of binary mixture was the aim of this work and the objective was to develop selective and time-saving spectrophotometric simultaneous assay methods for the cited drugs in their laboratory-prepared mixtures and their topical preparation without preliminary separation.

2. Experimental

2.1. Chemicals and reagents

Hydrocortisone acetate was obtained from October Pharma Company, Giza, Egypt. Its purity was certified to be 100.10±0.47 in accordance with BP [5]. Clioquinol was obtained from Kahira Pharmaceutical Company, Cairo, Egypt. Its purity was certified to be 99.35%±1.13 in accordance with USP [6]. Vioderm hydrocortisone cream was manufactured by Kahira Pharmaceutical Industries, Cairo, Egypt. It is composed of hydrocortisone acetate 1% and clioquinol 3%. Ethanol with HPLC grade was purchased from E. Merck, Germany.

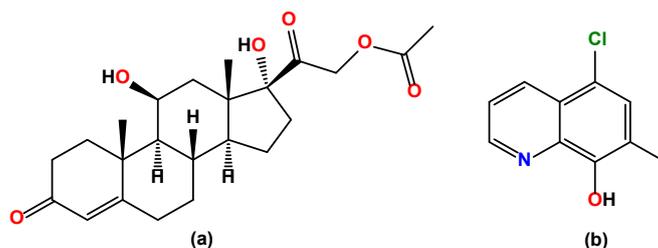


Figure 1. Structure of hydrocortisone acetate (a) and clioquinol (b).

2.2. Instrumentation

Spectrophotometric measurements were done by using a double-beam UV/Visible spectrophotometer model V-760 (Jasco, Japan) using Spectra manager® software. The absorption spectra of the solutions were carried out in a 1.00 cm quartz cell, at room temperature in the range from 200 to 400 nm.

2.3. Standard solutions

Stock standard solutions of HCA and CL (500 µg/mL) were made by dissolving 50 mg of each drug in ethanol in 100 mL volumetric flasks and then the volume was diluted to the mark using ethanol. Working standard solutions of HCA and CL (100 µg/mL) were prepared by dilution with the same solvent.

2.4. Procedures

2.4.1. Spectral characteristics

The absorption spectra of individual components and different mixtures at the range of 200-400 nm were scanned and stored.

2.4.2. Construction of calibration curves

Standard solutions corresponding to the concentration range 2-22 µg/mL of HCA and 1.5-7 µg/mL of CL were prepared by appropriate dilutions of working solutions with ethanol.

2.4.2.1. Absorbance subtraction method (AS)

The scanned spectra of 2-7 µg/mL of CL were recorded at 255 nm. The absorbance factor, which is a constant for pure drug, was estimated by taking the average of the ratios between the absorbance values of different concentrations of CL at λ_1 (isoabsorptive point, 243 nm) to those at λ_2 (331 nm). Calibration curve relates the absorbance of the absorption spectra (D^0) of HCA or CL at the isoabsorptive point 243 nm to the corresponding concentrations of HCA or CL to compute the unified regression equation.

2.4.2.2. Ratio subtraction method coupled with spectrum subtraction (RS-SS), constant multiplication (RS-CM), or constant value (RS-CV) methods

2.4.2.2.1. Ratio subtraction method

Calibration curve was constructed by plotting the absorbance of HCA at 242 nm versus the corresponding concentrations, then the regression equation was calculated.

2.4.2.2.2. Spectrum subtraction (SS) and constant multiplication (CM) methods

Calibration curve was constructed by plotting the absorbance of CL at 255 nm against the corresponding concentrations, then the regression equation was calculated.

2.4.2.2.3. Constant value method (CV)

The calibration curve was constructed by relating the measured amplitudes of ratio spectra (CL/CL') at the plateau region versus the corresponding concentration of CL, then the regression equation was calculated.

2.4.2.3. Derivative ratio method (DD^1)

The scanned absorption spectra of HCA were divided by the absorption spectrum of a standard solution of CL' (6 µg/mL) followed by recording the first order (D^1) of the resulting ratio spectra. The calibration curve relating the measured amplitudes at 242 nm versus the corresponding concentrations of HCA was constructed to compute the regression equation. The scanned absorption spectra of CL were divided by the absorption spectrum of a standard solution of HCA' (10 µg/mL) followed by recording the first order (D^1) of the resulting ratio spectra. The calibration curve relating the measured amplitudes at 253 nm and the corresponding concentrations of CL was constructed and the regression equation was calculated.

2.4.3. Analysis of laboratory-prepared mixtures

Aliquots of HCA and CL were accurately transferred from their working standard solutions into a series of 10 mL measuring flasks leading to mixtures with different ratios of the drugs under study. The spectra of the resulting mixtures were measured at 200-400 nm and recorded in the computer.

2.4.3.1. Absorbance subtraction method

In the laboratory mixtures, the absorbance of CL was obtained using the absorbance factor equation, while the absorbance of HCA was obtained by subtracting the absorbance of the CL from the total absorbance at 243 nm. The concentrations of HCA and CL were estimated from the unified regression equation at 243 nm.

2.4.3.2. Ratio subtraction coupled with spectrum subtraction (RS-SS), constant multiplication method (RS-CM) or constant value method

2.4.3.2.1. Ratio subtraction method

The absorption spectra of the laboratory-prepared mixtures were divided by the spectrum of CL' (6 µg/mL) as a divisor, then subtracting the amplitudes in the plateau region at λ 325-350 nm (the constant) from that ratio spectrum. The zero order spectra of HCA were resolved via multiplying the resulted ratio spectra by the divisor (CL'). The concentration of HCA was computed using the corresponding regression equation at 242 nm.

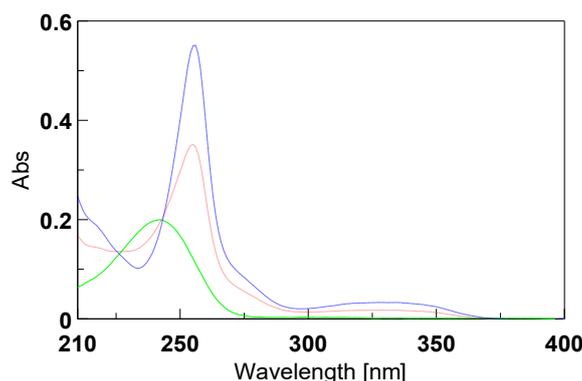


Figure 2. Zero-order spectra of HCA (4 µg/mL) (—) and CL (4 µg/mL) (—•—), separately in ethanol and binary mixture of HCA and CL, (2 µg/mL) of each (—•—) showing isoabsorptive points at 243 nm.

2.4.3.2.2. Spectrum subtraction and constant multiplication methods

The absorption spectra of CL were obtained through CM by multiplying the obtained constant value by the spectrum of CL' (6 µg/mL) as a divisor. Alternatively, CL spectra could be obtained via SS by subtracting two spectra from each other; the obtained spectra of HCA from the spectra of the corresponding binary mixtures. The concentration of CL in each laboratory-prepared mixture was estimated using the corresponding regression equation at 255 nm.

2.4.3.2.3. Constant value

The amplitudes of the ratio spectra of laboratory prepared mixtures (Lab/CL') at the plateau region were measured and the concentrations of CL were calculated from the corresponding regression equation.

2.4.3.3. Derivative ratio method

The same procedures under construction of calibration curves were applied and the concentrations of HCA and CL were calculated from the corresponding regression equations.

2.4.4. Application to the topical preparation

Vioderm hydrocortisone cream (1 g) was weighted in a beaker and stirred with 50 mL of extracting solvent ethanol on a water bath at 75 °C for 60 min. The dissolved solution was transferred into 100 mL volumetric flask and complete extraction of the drugs from the residue was accomplished using additional 10 mL ethanol and stirring for 15 minutes. The obtained solution was mixed for 10 min by vortex shaker. The volume of the flask was completed to the mark with ethanol to get the claimed concentrations in the dosage form (100 µg/mL of HCA and 300 µg/mL of CL). The obtained solution was filtered, and 20 mL of the solution was accurately transferred into 100 mL volumetric flask then the volume was completed to the mark with ethanol to obtain a working solution having a concentration of 20 µg/mL of HCA and 60 µg/mL of CL. 1 mL of the working solution was accurately transferred into 10 mL volumetric flask and the volume was completed with ethanol to obtain a final concentration of 2 and 6 µg/mL of HCA and CL, respectively. The concentrations of the studied drugs were calculated from the corresponding regression equations, using the procedures mentioned under analysis of laboratory-prepared mixtures.

3. Results and discussion

The objective of this work was to determine the concentration of HCA and CL accurately and specifically in their bulk powders, lab mixtures, and pharmaceutical dosage forms by various spectrophotometric methods. By scanning the absorption spectra of HCA and CL in ethanol, there was an overlap between the spectral bands in the wavelength region of 210-275 nm, which prevents the direct assay of both drugs (Figure 2), so different spectrophotometric methods were applied to have good resolution and simultaneous determination of each drug without any preliminary steps.

3.1. Absorbance subtraction method

The AS method could be used for the analysis of a binary mixture with overlapped spectra with intersecting at an isoabsorptive point (λ_{iso}), in which one of the two drugs is more extended than the other and does not show any contribution at another wavelength (λ_2) [16]. Hydrocortisone acetate and Clioquinol are presented in their dosage form in the proportion 1:3, where the absorption spectra of both in ethanol show an overlap and an isoabsorptive point at 243 nm. This was verified experimentally by scanning the absorbance spectra of 4 µg/mL of HCA and CL separately in ethanol and in the binary mixture of HCA and CL 2 µg/mL each (Figure 2). The total concentration of both drugs could be calculated at this isoabsorptive point as the two drugs have the same absorptivity and act as one component at this point.

The value of absorbance factor of pure CL represents the average ratio between the absorbance at two wavelengths, one of these wavelengths is λ_{iso} , while the other wavelength λ_2 shows no contribution from HCA. The absorbance of HCA in the binary mixture was calculated by subtracting the absorbance due to CL contribution from the total absorbance at λ_{iso} .

$$\text{Abs. of CL in the mixture at } \lambda_{iso} = \text{Abs. factor of pure CL} \times \text{Abs}_{\lambda_2} \quad (1)$$

$$\text{Abs. of HCA in the mixture at } \lambda_{iso} = \text{Abs}_{\lambda_{iso}} (\text{HCA+CL}) - [\text{Abs. factor of pure CL} \times \text{Abs}_{\lambda_2}] \quad (2)$$

where the absorbance factor is obtained from the average ratio of absorbance of pure CL at λ_{iso} to its absorbance at λ_2 (where no contribution of HCA)

Quantitative determination of HCA and CL were estimated using the corresponding unified regression equation, which was gotten by constructing a calibration curve between the absorbance spectra of HCA or CL at λ_{iso} versus the corresponding concentrations.

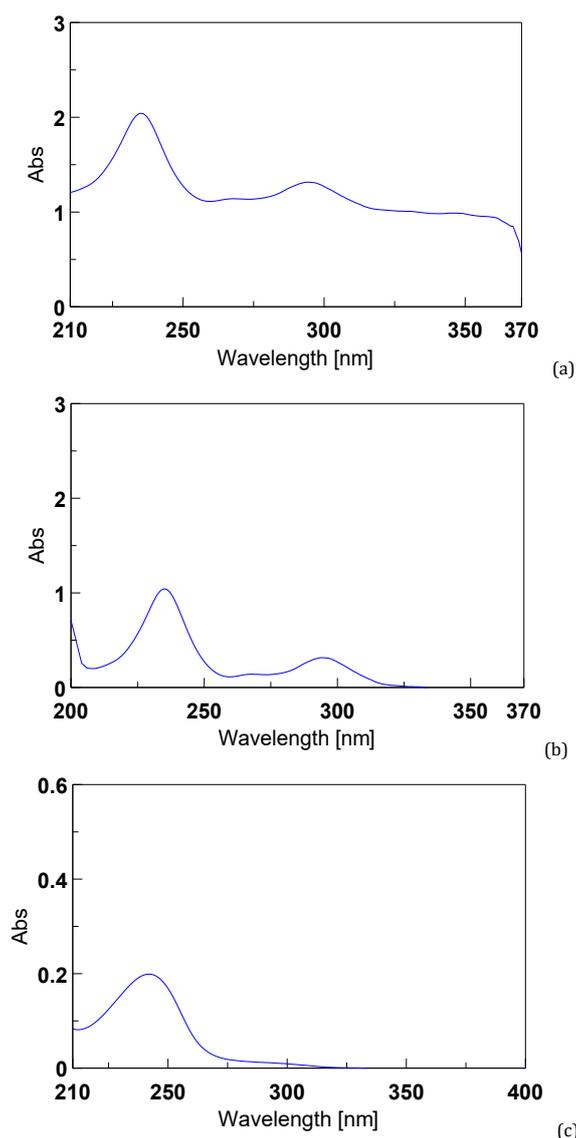


Figure 3. (a) Ratio spectra of a mixture of 4 µg/mL HCA and 6 µg/mL CL using CL' (6 µg/mL) as a divisor. (b) subtracting the value of the constant from the ratio spectra (c) the obtained HCA spectrum.

3.2.1. Ratio subtraction method

This method could be used for the analysis of binary mixtures in which the spectrum of one drug is more extended than that of the other one [17,18]. It was applied to solve the overlapping spectra of the mixture of HCA and CL to get the less extended (HCA) in zero order (Figure 2). The method involves dividing the zero-order spectrum of the mixture by the spectrum of a divisor which is known concentration of CL' (6 µg/mL). The resulted ratio spectrum is a new graph that represents $\left(\frac{HCA}{CL'} + \text{constant}\right)$. By subtracting this constant (plateau in 325-350 nm), after that multiplying the new graph by the divisor, the original zero-order spectrum of HCA in the mixture can be obtained. Thus, the interference of CL was removed (Figure 3).

3.2.2. Constant multiplication and spectrum subtraction methods

These methods are complementary to the ratio subtraction method to get the more extended drug [19,20]. The absorption

spectra of CL could be obtained by constant multiplication method via multiplying the constant value which obtained from ratio spectra by the divisor CL' (6 µg/mL) (Figure 4).

In addition, the absorption spectra of CL were obtained via spectrum subtraction through subtracting the two spectra from each other; the obtained spectra of HCA from the spectra of the corresponding binary mixtures (Figure 5). The concentration of CL in each mixture was calculated using the corresponding regression equation at 255 nm.

3.3. Derivative ratio (DD¹) method

The method was used for simultaneous determination of the compounds to resolve the severely overlapped absorption spectra in binary or ternary mixtures [21-23]. DD¹ spectrophotometric method was used to increase the selectivity of the analysis of HCA without interference from CL. One of the main advantages of DD¹ method over the traditional derivative method (D¹) is that we can cancel the whole spectrum of the interfering substance.

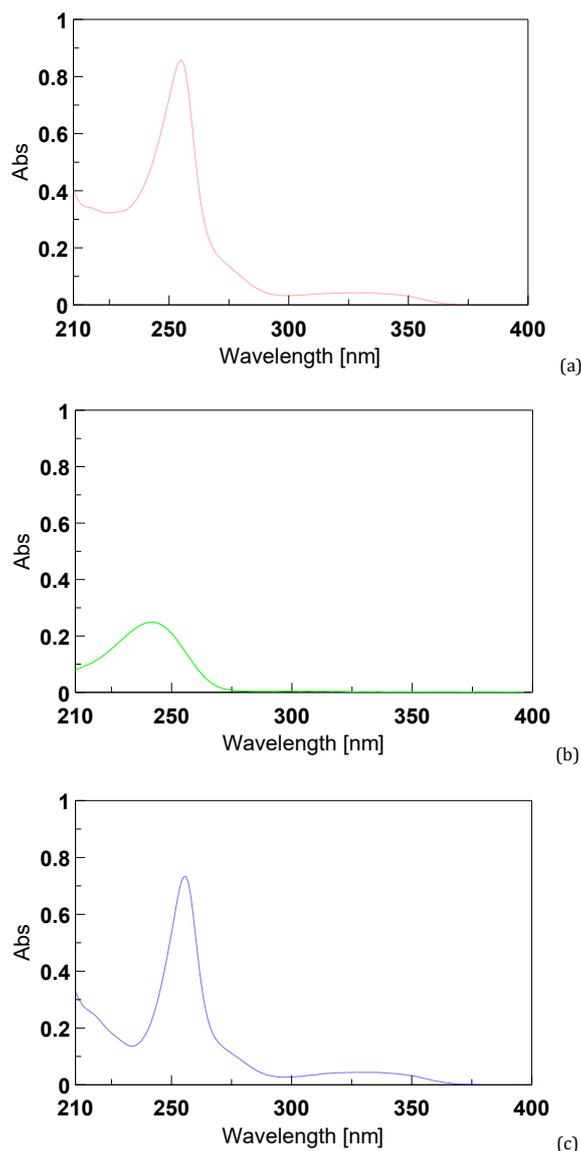


Figure 4. (a) Absorption spectra of a binary mixture of HCA and CL, 5 µg/mL each, (b) the obtained absorption spectra of HCA from ratio subtraction method, (c) the obtained absorption spectra of CL after subtraction.

For optimizing the DD¹ method, many concentrations of the CL as a divisor were tried including 1, 3, 4, and 6 µg/mL of CL and the best results were achieved by 6 µg/mL of CL as a divisor (Figure 6a). The obtained ratio spectra ($\frac{HCA}{CL}$) were differentiated according to the wavelength used, and DD¹ values showed good linearity and precision at 242 nm (Figure 6b).

For determination of CL in the presence of HCA, many concentrations of HCA as a divisor were, tried including 2, 5, 10, and 20 µg/mL of HCA, and the best results were achieved by using 10 µg/mL of the HCA as a divisor (Figure 6c). The obtained ratio spectra ($\frac{CL}{HCA}$) were recorded at 253 nm (Figure 6d).

3.4. Comparative study

The AS method has the advantage that both drugs in their binary mixture were assayed using a unified regression equation at λ_{iso} in comparative with the previously established isoabsorptive point method which could determine the total concentration of two drugs while one of the drugs were measured by using other spectrophotometric method as a

complementary method. The main disadvantage is it requires an isoabsorptive point with the extension of the spectrum of one component over the other one. Another disadvantage is the multiple manipulation steps and increased probability of error in calculating the absorbance factor especially in low concentrations.

Among the advantages of RS-SS and RS-CM over DD¹, is minimum manipulation step where both drugs can be determined using a single divisor in contrast to DD¹ method in which two divisors should be used for getting both drugs. In addition, the whole spectrum of the interfering substance is eliminated. We can obtain the spectra of pure components which confirm the spectral profile of each component of interest and allow the determination of both components at their λ_{max} giving better accuracy and reproducibility.

The CV method has lower manipulation steps than AS in the determination of the more extended spectrum, and it can be used for the analysis of binary and ternary mixtures. Its limitation upon analysis of mixtures containing low concentrations of the extended component, where the calculation of the constant value through plateau region was inaccurate due to the low signal to noise ratio.

Table 1. Validation parameters and obtained results of determination of pure samples of HCA and CL by the proposed method.

Parameter	AS	RS-SS	RS-CM	RS-CV	DD ¹	
	HCA/CL	HCA	CL	CL	HCA	CL
Accuracy ^a	100.29±1.2	99.67±1.01	99.41±0.51	99.92±1.07	99.46±1.01	99.38±0.55
Precision						
Repeatability ^b	0.488	1.174	0.392	0.304	0.348	0.524
Intermediate precision ^c	0.893	1.567	0.724	0.603	1.038	0.587
Linearity ^d						
Slope	0.0568	0.0463	0.1662	0.1994	0.0159	0.0492
Intercept	0.02	0.0164	-0.1072	-0.1985	0.0056	-0.0264
Mean ±SD	100.03±0.50	100.01±0.44	99.87±0.83	100.04±0.66	99.74±0.79	100±0.85
Range (µg/mL)	2-7	2-22	1.5-7	1.5-7	2-22	1.5-7

^aThe accuracy (n = 5), mean recovery of five concentrations (7, 8, 12, 17, 21 µg/mL) for HCA and (2.5, 3.5, 4.5, 5.5, 6.5 µg/mL) for CL.

^bThe intraday (n = 3), RSD of three concentrations (5, 10, 15 µg/mL) for HCA and (3, 5, 7 µg/mL) for CL repeated three times within day.

^cThe interday (n = 3), RSD of concentrations (5, 10, 15 µg/mL) for HCA and (3, 5, 7 µg/mL) for CL repeated three times in three days.

^dSix calibration points, average of three experiments.

Table 2. Determination HCA and CL in laboratory prepared mixtures by the proposed spectrophotometric method.

Concentration (µg/mL)		Recovery % ^a							
AS		RS		RS-SS		RS-CV		DD ¹	
HCA	CL	HCA	CL	HCA	CL	CL	CL	HCA	CL
2	4	101.64	100.84	100.05	99.51	99.46	100.65	99.46	98.88
2	6	99.60	99.58	100.07	98.89	98.89	98.83	100.29	99.78
4	6	101.21	101.51	100.2	99.69	99.6	99.76	100.29	101.84
5	5	99.15	100.70	100.37	100.95	100.95	100.08	99.96	102.58
6	3	98.37	99.57	99.08	99.77	99.77	100.93	98.63	98.21
Mean±SD		99.99±1.39	100±0.85	100.05±0.82	99.73±0.76	99.76±0.75	99.95±0.50	99.39±1.02	99.97±1.40

^aAverage of three determinations.

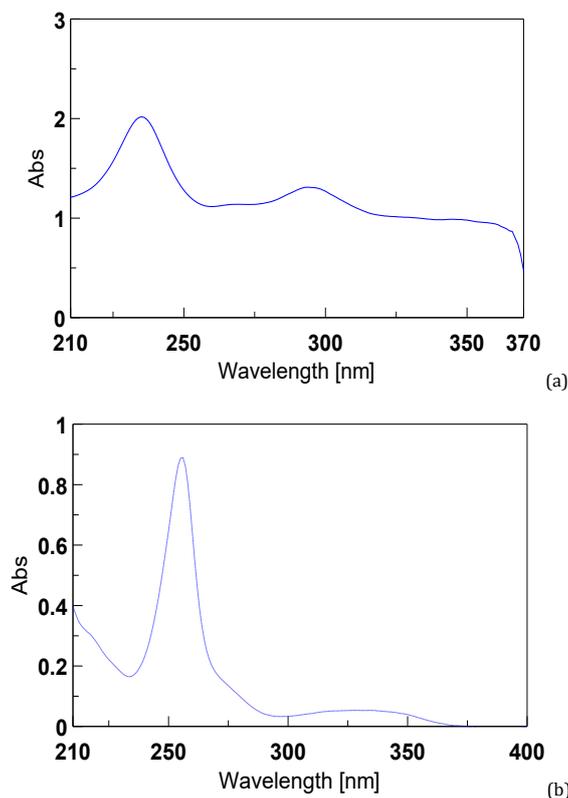


Figure 5. (a) Ratio spectra of a mixture of 4 µg/mL of HCA and 6 µg/mL of CL using CL' (6 µg/mL) as a divisor. (b) CL spectra obtained from multiplying the constant value by the spectrum of the divisor.

In DD¹ method, the main advantage is that the entire spectrum of the interfering drug can be cancelled. Also, the selection of the wavelength for calibration is not critical as in the other methods, and it can measure at the maximum or minimum peak amplitude. Its limitations are multiple manipulation steps, decreasing signal-to-noise ratio and the selection of the divisor is critical to increase sensitivity and decrease noise.

Validation of the developed methods was achieved in accordance to ICH guidelines [24]; where the parameters were

presented in Table 1. The methods showed good accuracy and precision. The specificity was assessed by the analysis of binary mixtures containing different proportions of the drugs and the methods were shown to be specific as shown in Table 2. The proposed methods were applied for the determination of the cited drugs in Vioderm hydrocortisone cream and the results were compared statistically with the reported HPLC method [15] as shown in Table 3.

Table 3. Statistical comparison between the results obtained by the proposed methods and the reported method [15] for the determination of HCA and CL, in pharmaceutical dosage form.

Drug name	HCA				CL					
	AS	RS	DD ¹	Reported method ^a	AS	SS	CM	CV	DD ¹	Reported method ^a
Vioderm hydrocortisone ^{®b} (found%±SD)	100.64± 1.63	100.18± 0.81	100.52± 0.79	99.58± 0.89	98.80± 0.92	99.33± 0.62	98.58± 0.24	98.78± 1.14	98.72± 1.11	99.54± 0.99
Variance	2.650	0.651	0.626	0.786	0.855	0.387	0.059	1.293	0.223	0.988
Student's t test ^c (4.303)	0.511	0.534	0.813		0.901	0.531	0.158	0.777	0.787	
F value ^c (19)	3.371	1.207	1.255		1.156	2.549	16.644	1.308	1.237	

^a C18 Hypersil ODS column (Shandon) using a mobile phase consisting of methanol - 0.05 M phosphoric acid (80:20, v: v) at a flow rate of 2 mL/min and UV detection at 240 nm.

^b n=3.

^c The values in the parenthesis are the corresponding theoretical values of t and F at p = 0.05.

Table 4. Statistical analysis of the proposed methods compared to the official [5] and reported method [10] of HCA and CL, respectively, in their pure powdered form.

Drug name	HCA				CL					
	AS	RS	DD ¹	Official method ^a	AS	SS	CM	CV	DD ¹	Reported method ^b
Mean ^c	100.03	100.44	99.92	100.10	100.03	100.95	100.95	100.04	99.82	99.1
±SD	±0.50	±0.44	±0.11	±0.47	±0.50	±1.47	±1.47	±0.66	±0.64	±1.02
Variance	0.2511	0.1970	0.1161	0.2213	0.2511	2.1609	2.1609	0.4308	0.4076	1.0311
Student's t test ^d (4.303)	0.712	0.039	0.431		0.092	0.121	0.121	0.174	0.105	
F test ^d (5.05)	1.135	1.124	1.440		4.105	2.0958	2.0958	2.859	2.530	

^a Spectrophotometric method at 241.5 nm for HCA.

^b HPLC method using methanol-acetonitrile (1:1) as a mobile phase for CL.

^c n=6

^d The values in the parenthesis are the corresponding theoretical values of t and F at p = 0.05.

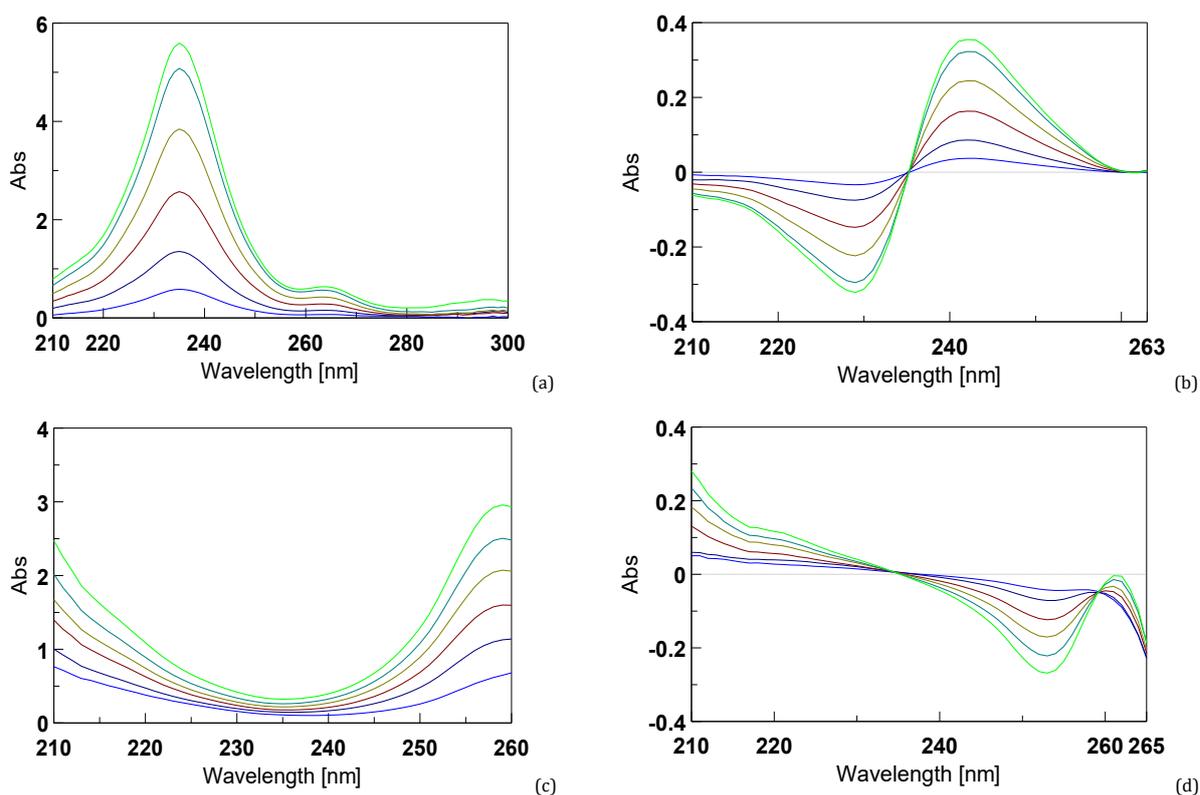


Figure 6. (a) The ratio spectra of HCA, (b) DD1 of HCA using the spectrum of CL' (6 µg/mL) as a divisor, (c) The ratio spectra of CL, and (d) DD1 of CL using the spectrum of HCA' (10 µg/mL) as a divisor.

The proposed methods for analysis of drugs in pure powder were also statistically compared to those of the official [5] and reported HPLC [10] methods showing no significant difference as presented in Table 4.

4. Conclusion

In this work, different simple and accurate spectrophotometric methods were applied for the simultaneous determination of binary mixtures with good accuracy and accepted

precision. The proposed methods were sensitive and specific with minimum mathematical manipulation steps. They could be simply applied in quality control laboratories without the need of any sophisticated software. The proposed methods were applied to assay the cited drugs either in their pure bulk powders, laboratory prepared mixtures, or in their pharmaceutical formulation without any preliminary separation steps.

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Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.

Author contributions: All authors contributed equally to this work.

Ethical approval: All ethical guidelines have been adhered.

Sample availability: Samples of the compounds are available from the author.

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