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ORIGINAL ARTICLE

Development and validation of stability indicating TLC densitometric and spectrophotometric methods for determination of Clobetasol propionate

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KEYWORDS

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TLC-densitometry;
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Alkaline degradation products

Abstract Two simple analytical techniques that manipulate the inherent spectroscopic properties of the drug differently were developed for Clobetasol propionate (CP) determination in the presence of its alkaline hydrolytic degradation products. The first method depends on TLC-densitometric determination of the UV–visualized bands after TLC separation of CP in the presence of its alkaline degradation products in its bulk and pharmaceutical formulation. Separation was performed on preactivated silica gel 60 F_{254} TLC plates using ethyl acetate:hexane:ammonia (5:5:0.2, by volume) as a developing system followed by scanning at 240 nm. Linear correlation was obtained in the range of 0.10–0.50 μg/band. The second method was ratio difference spectrophotometry. It was applied by measuring the difference in peak amplitude of the ratio spectra at 243.40 and 256.40 nm. The selectivity of both methods was checked by analyzing laboratory prepared mixtures containing different concentrations of CP and its alkaline degradation products. The methods were validated in compliance with ICH guidelines. The methods determined CP in its bulk powder with average percentage recoveries of 99.60% ± 1.09 and 99.44% ± 1.60 for densitometry and ratio difference, respectively. Both methods were successfully applied for quantification of CP in its commercial cream.

1. Introduction

Clobetasol propionate is a class 1 corticosteroid. It is a super-potent synthetic di-halogenated analog of prednisolone.[1–3] It is 1800 times more potent than hydrocortisone[1] and is currently

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the most potent topical corticosteroid available in the market.[2,4–8] Clobetasol propionate possesses potent anti-inflammatory,[9,10] anti-pruritic and vaso-constrictive action.[7] It is used in the treatment of skin conditions such as severe psoriasis, seborrhoeic dermatitis, and extreme photodermatitis in HIV/AIDS patients.[11,12]

Clobetasol propionate is 21-chloro-9α-fluoro-11β, 17α-dihy droxy-16β-methylpregna-1,4-diene-3,20-dione 17-propionate,[13–16] as shown in Fig. 1. Containing an ester linkage, Clobetasol propionate is susceptible to hydrolysis to its corresponding acid and alcohol.

Clobetasol propionate was analyzed using spectrophotometry,[17,18] HPTLC[19–21] and HPLC.[22–25] The Clobetasol propionate degradation products were not identified in any of the stability indicating studies that were conducted for CP determination according to our knowledge.

The International Conference on Harmonization (ICH) Q1A (R2)[26] guidelines entitled "stability testing of new drugs substances and products" require the stress testing to be carried out to elucidate the inherent stability characteristics of the drug substance. A stability indicating method is a specific method that quantifies the standard drug alone and in the presence its degradation products. The information obtained from the stability study may help to establish degradation patterns during drug product development and the validation of analytical procedures.

The aim of this work was to identify the hydrolytic degradation products of CP and to develop validated stability-indicating methods for the determination of CP in the presence of its degradation products to be used for quality control analysis of CP in bulk form and in its pharmaceutical formulation.

2. Experimental

2.1. Instruments

- Camag-Linomat 5 autosampler (Switzerland). Camag microsyringe (100 μL). Camag TLC scanner 3 S/N 130319 with winCATS software, the following requirements are taken into consideration:
  - Slit dimensions: 5 mm × 0.2 mm.
  - Scanning speed: 20 mm/S.
  - Spraying rate: 10 μL s^{-1}.
  - Data resolution: 100 μm/step.
- Precoated TLC-plates, silica gel 60 F254 (20 cm × 20 cm, 0.25 mm), E. Merck (Darmstadt-Germany). PH-meter, Digital pH/MV/TEMP/ATC meter, Jenco Model-5005 (USA).

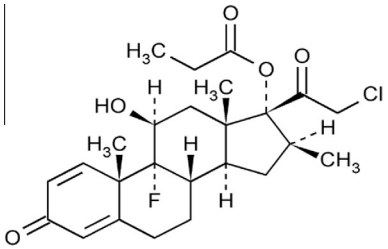


Figure 1 Chemical structure of clobetasol 17-propionate ($C_{25}H_{32}ClFO_5$, MW = 467.0).

- A double beam UV–visible spectrophotometer (SHIMADZU, Japan), model UV-1800 PC with matched 1 cm quartz cells, connected to an IBM-compatible PC and an HP-600 inkjet printer. Bundled, UV-PC personal spectroscopy software version 2.43 was used to process the absorption and the derivative spectra. The spectral band width was 2 nm with wavelength-scanning speed of 2800 nm min^{-1}.

2.2. Materials and reagents

2.2.1. Pure standard

Clobetasol propionate, working standard, was kindly supplied by GlaxoSmithKline Beecham, Cairo, Egypt. Its purity was certified to be 100.00% according to a reported HPLC method.[24]

Chlorocresol, working standard, was kindly supplied by, GlaxoSmithKline Beecham Cairo, Egypt. Its purity was found to be 100.00% according to its official USP method.[14]

2.2.2. Pharmaceutical formulation

Dermovate® cream is a trade mark of GlaxoSmithKline group companies, manufactured by SmithKline Beecham, Egypt. Batch No. H101072, each 100 g cream was labeled to contain 0.05 g of CP.

2.2.3. Degraded sample

Degradation products were prepared under alkaline stress condition by dissolving 100 mg of pure CP in the least amount of methanol, refluxing with 10 mL 0.1 N NaOH solution for 4 h at temperature 100 °C. The degradation process was followed by TLC through the disappearance of the spot corresponding to the drug and appearance of two new spots corresponding to the formed degradation products using ethyl acetate:hexane: ammonia (5:5:0.2, by volume) as a developing system. After complete degradation, the solution was neutralized using 0.1 N HCl, evaporated nearly to dryness and the degradation products were extracted with methanol 20 mL × 4 times and completed to 100 mL with methanol. Part of the mixed degradation product solution was separated by preparative TLC then scratched from the plate and extracted with methanol to identify the degradation products.

2.2.4. Chemicals and reagents

All chemicals and reagents used throughout this work, were of pure analytical grade.

Sodium hydroxide and hydrochloric acid, 0.1 N aqueous solutions, hexane, ethyl acetate, and ammonia, 33% solution; El-Nasr Pharmaceutical Chemicals Co., (Abu-Zabaal, Cairo, Egypt). Methanol; spectroscopic grade; S.D. Fine-Chem Limited, (Mumbai, India). Methanol, HPLC-grade; E. Merck (Darmstadt, Germany) and HPLC grade water.

2.3. Standard solutions

Stock standard solution of CP 0.1 mg mL^{-1} in methanol for both TLC-densitometric and spectrophotometric methods.

Stock standard solution of the alkaline degradation products derived from complete degradation of 100 mg CP to prepare 0.1 mg mL^{-1} standard solution of CP in methanol.

Stock standard solution of Chlorocresol 0.1 mg mL^{-1} in methanol for ratio difference spectrophotometric method.

All stock standard solutions were freshly prepared on the day of analysis and stored in refrigerator to be used within 24 h.

3. Procedures

3.1. Construction of calibration curve for TLC-densitometric method

Aliquots equivalent to 0.10–0.50 mg from CP standard solution (0.1 mg mL^{-1} in methanol) were transferred separately, into a series of 10-mL measuring flask and the volume was completed with methanol. Ten micro liters of each solution were applied in the form of bands on a TLC plate using a Camag-Linomat IV applicator.

The bands were applied 14 mm apart from each other and 15 mm from the bottom edge of the plate with a length of 6 mm. Linear ascending development was performed in a chromatographic tank previously saturated with ethyl acetate:hexane:ammonia (5:5:0.2, by volume) for 30 min at room temperature. The developed plates were air-dried, then scanned at 240 nm using deuterium lamp. A calibration curve relating the optical density of each band to the corresponding concentration of CP was constructed. The regression equation was then computed for the studied drug and used for determination of samples containing unknown concentrations of CP.

3.2. Construction of calibration curve for ratio difference spectrophotometric method

Aliquots of CP stock solution (0.1 mg mL^{-1}) equivalent to 25–250 μg were accurately transferred into a series of 10-mL volumetric flasks then diluted to volume using methanol. The absorption spectra of these solutions and the alkaline degradation products were divided by the absorption spectrum of 10 μg mL^{-1} Chlorocresol (as a divisor) where the contribution of the alkaline degradation products was removed. Calibration graphs were constructed relating the difference in amplitude at 243.40 and 256.40 nm to the corresponding concentrations of CP. The regression equation was then computed for the studied drug at the specified wavelengths and used for determination of samples containing unknown concentrations of CP.

3.3. Analysis of laboratory prepared mixtures containing different ratios of Clobetasol propionate and its alkaline degradation products using the suggested methods

Aliquots of intact drug and degradation products were mixed to prepare different mixtures containing 10–50% (for TLC method) and 2.5–30% of the degradation products (for ratio difference method) then the procedures were completed as mentioned under each method. The concentrations were calculated from the corresponding regression equations.

3.4. Assay of pharmaceutical formulations (Dermovate® cream)

Accurate weights of cream (0.60 g in 8 mL acetone for TLC method and 0.20 g in 7 mL methanol for ratio difference method) were melted in water bath at 60–70 °C, then magnetically stirred for 30 min, cooled in a refrigerator and filtered. The clear filtrates were collected in separate 10 mL volumetric flasks. Appropriate volume of solvent (acetone for TLC method and methanol for ratio difference method) was added to prepare the final concentration equivalent to 30 and 10 μg mL^{-1} of CP for TLC and spectrophotometric determinations, respectively.

4. Results and discussion

In pharmaceutical practice, quality means that the product to be handled should be safe and effective. Stability studies demonstrate that the necessary critical characteristics present at the time of the release can be expected to be present when the dosage form is administered. If safety and efficacy values decline, stability studies are the main judge that determines when the product should be withdrawn from the market.

The ICH[26] guidelines Q1A (R2) on "Stability Testing of New Drug Substances" suggest that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and efficacy must be done by validated stability indicating methods. Forced degradation was reported for CP, it was found that the drug degrades under basic, oxidative, and light conditions.[24] However the degradation products have not been identified. The structural characterization and synthesis of the degradation products allow both to establish the degradation pathways and also

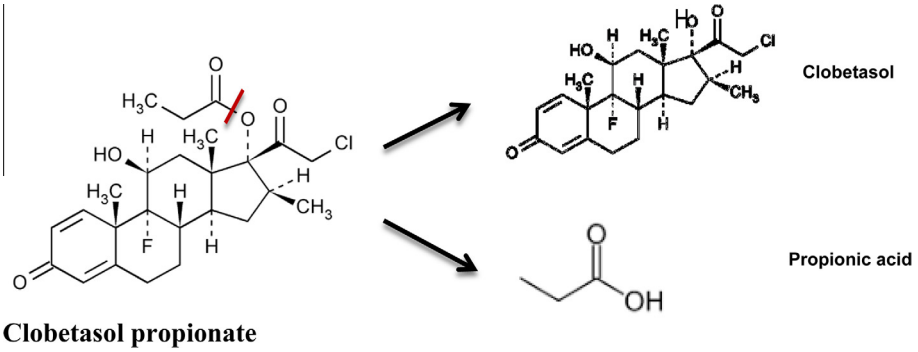
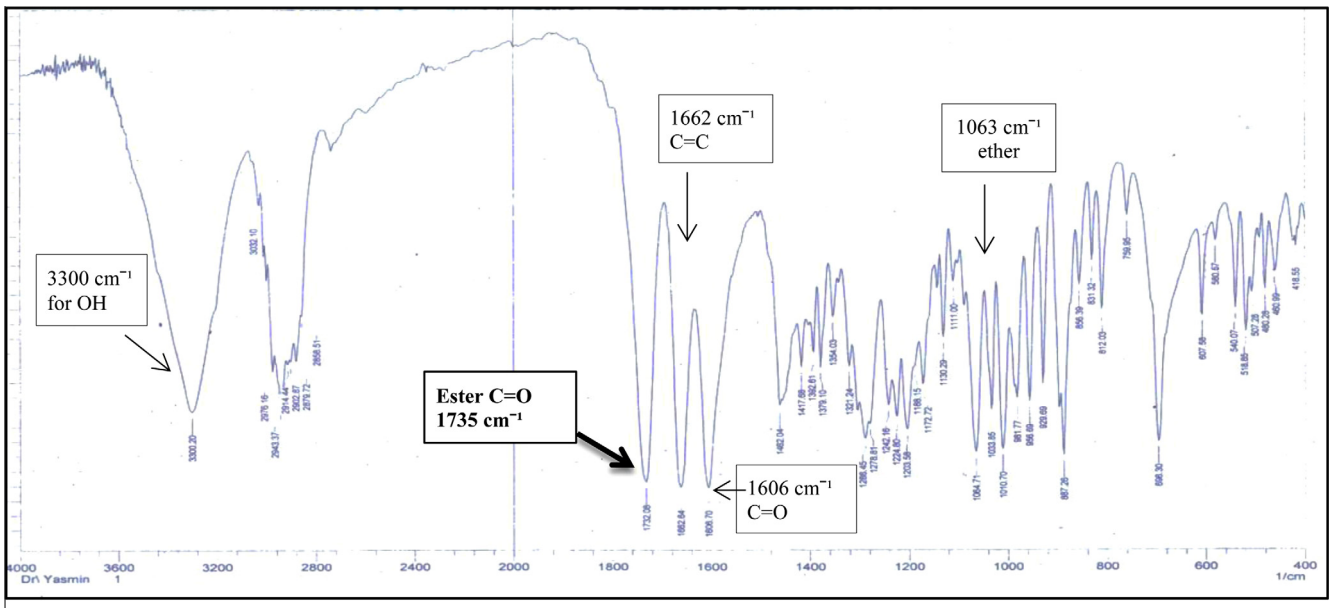
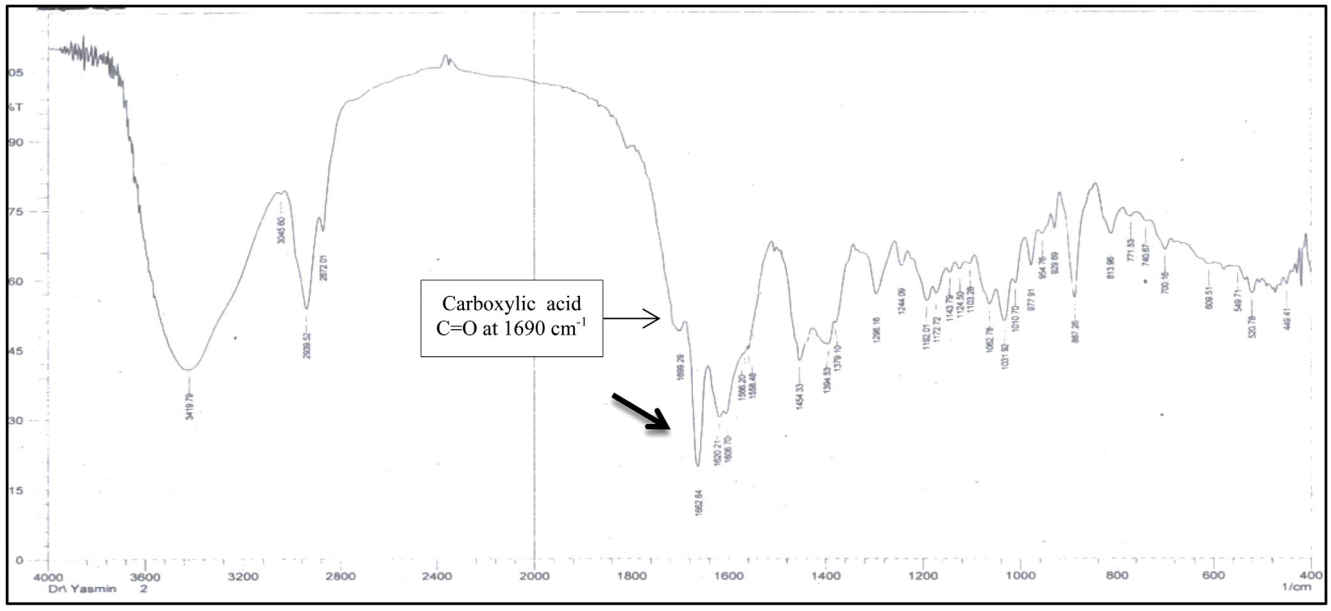


Figure 2 Suggested degradation products result from cleavage of ester bond present in Clobetasol propionate to the corresponding alcohol and acid.



(a)



(b)

Figure 3 (a) IR spectrum of Clobetasol propionate. (b) IR spectrum of Clobetasol propionate alkaline degradation products.

their quantitative determination in drug substance and product. Hence, in the present work the chemical degradation pathway of CP was established and selective, precise and accurate stability-indicating methods for the determination of CP in the presence of its degradation products was also developed.

However, a recently published HPTLC method[19] studied the stability behavior of CP under different stress conditions. But that method didn't suggest the degradation pathway and it didn't isolate or identify the separated degradation products under the studied conditions. Also the described methods in this study have many advantages over that HPTLC method as working in a wider range for CP determination, which indicates the high sensitivity of the methods. The reported method also used more prolonged conditions, which results in dramatic degradation of the drug that does not occur in reality.

In this work, stress testing was carried out on CP using alkaline hydrolysis; it was subjected to 0.1 N NaOH for 4 h at 100 °C. The degradation process under the previously mentioned conditions was followed up using TLC and the compound was found to be liable to degradation under these conditions giving two degradation products, as shown in Fig. 2. The First degradation product was suggested to be Clobetasol, which is one of the CP impurities stated in BP (impurity G),[13] which will affect the topical: systemic potency ratio

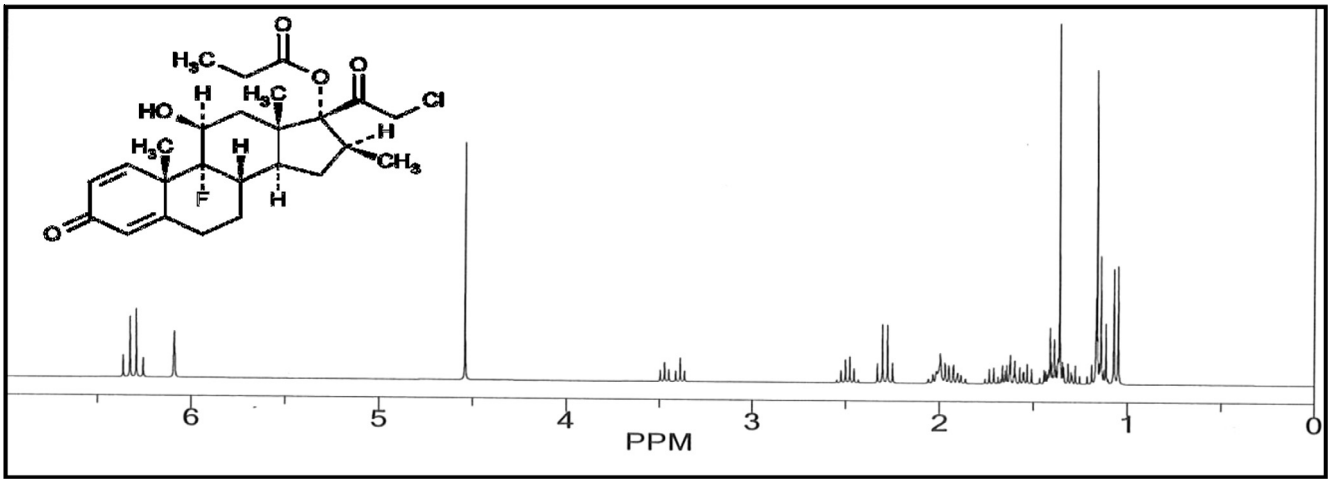


Figure 4 ^{1}H NMR spectrum of Clobetasol propionate.

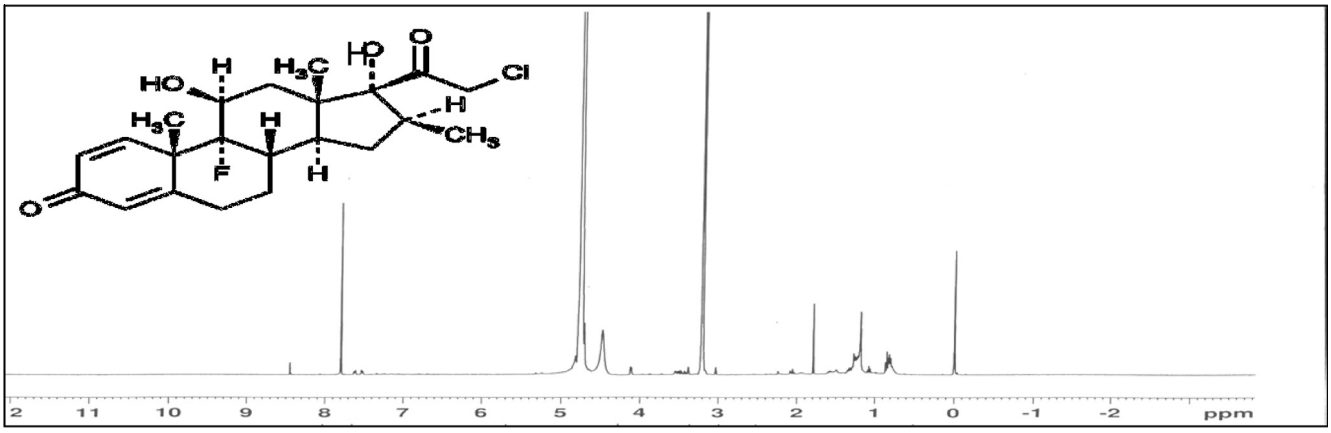


Figure 5 ^{1}H NMR spectrum of the first Clobetasol propionate degradation product suggested to be Clobetasol, using methanol as solvent.

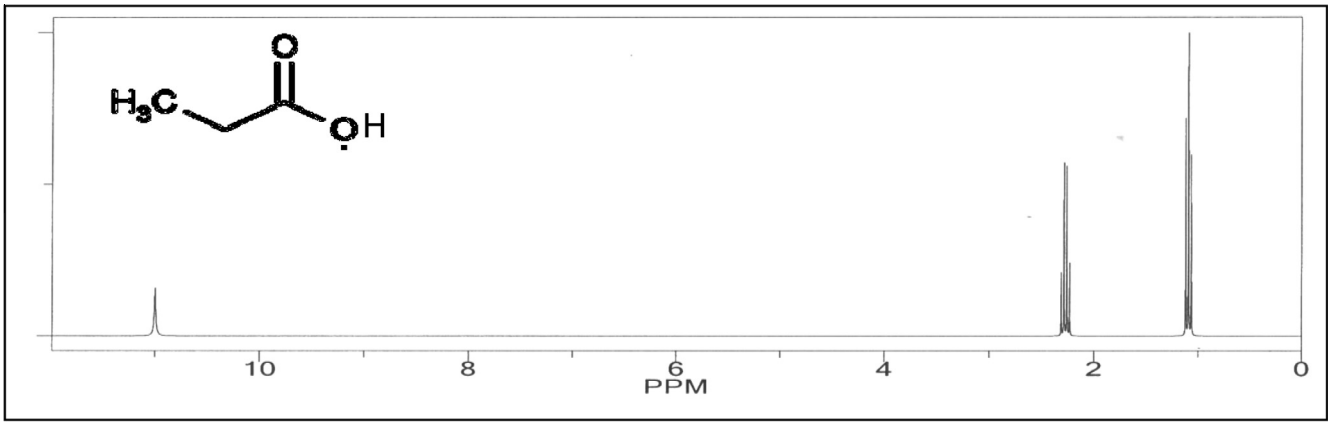


Figure 6 ^{1}H NMR spectrum of the second Clobetasol propionate degradation product suggested to be Propionic acid, using methanol as a solvent.

of the drug and increase its systemic effect. In order to achieve local glucocorticoid activity and minimize CP systemic effects, the corticosteroid skeleton was modified by esterification at C-17 and produces an analog that is rapidly inactivated after its systemic absorption.[27] So, it is essential to develop analytical procedure which will serve as a reliable, accurate and sensitive

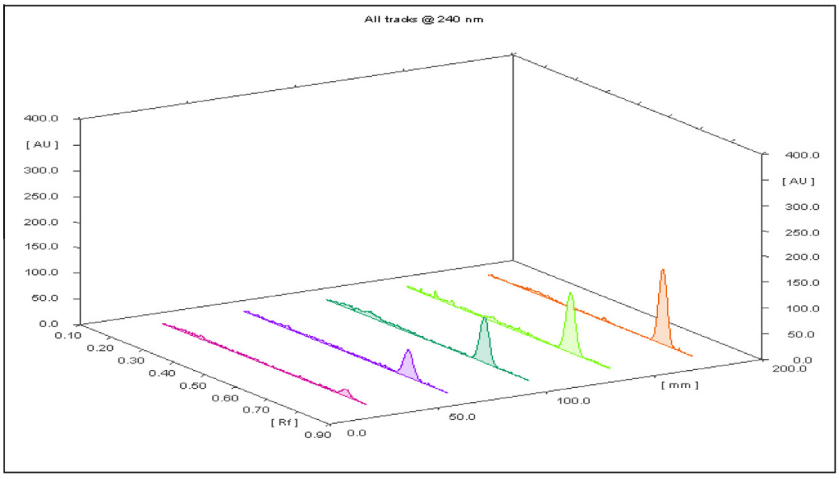


Figure 7 (3D) Densitogram of Clobetasol propionate (R_f = 0.63) in the concentration range (0.10–0.50 μg/band), at 240 nm using ethyl acetate:hexane:ammonia (5:5:0.2, by volume) as a developing system.

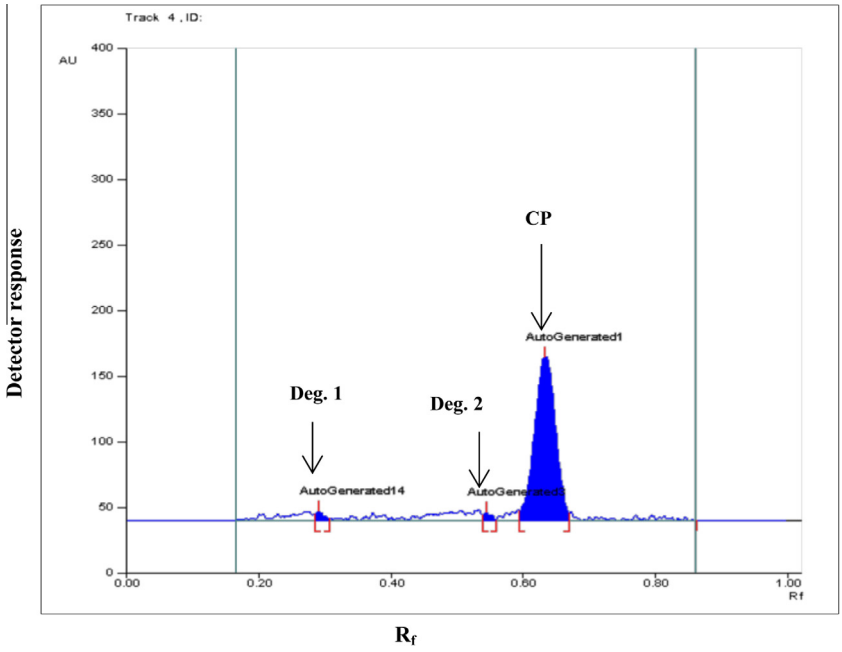


Figure 8 TLC chromatogram of Clobetasol propionate (0.45 μg/band, R_f = 0.63) and its alkaline degradation products (Deg. 1 & 2) (0.50 μg/band, R_f = 0.30 & 0.56).

Table 1 Parameters of system suitability of the developed TLC-densitometric method for the determination of Clobetasol propionate in the presence of its degradation products.

Parameters	Degradation product (1)	Degradation product (2)	Clobetasol propionate	Reference value[29]
Resolution (R_s)	7.20	1.73		> 1
Separation factor (α)	2.95	1.34		> 1
Tailing factor (T)	1.00	1.00	1.00	= 1 for typical symmetric peak
Capacity factor (K′)	2.33	0.79	0.59	The higher the capacity factor, the longer the retention factor

stability indicating method for the determination of CP in the presence of its hydrolytic degradation products. The second degradation product was suggested to be propionic acid.

These claims were confirmed when the degradation products were identified using IR and ^{1}H NMR spectrometry. The IR spectrum of CP (Fig. 3a) shows a band corresponding to the ester carbonyl group stretching at 1735 cm^{-1} which disappeared in the IR spectrum of degradation products (Fig. 3b). The absence of this characteristic band is an evidence of the cleavage of the ester bond.

The degradation products were identified separately by ^{1}H NMR spectrometry using methanol as a solvent. By comparing the NMR spectrum of both CP (Fig. 4) and Clobetasol (Fig. 5), it shows similar signals at both NMR spectra except the signals at 2.29 and 1.14 ppm corresponding to aliphatic protons in CH_2 and CH_3, respectively. The disappearance of these signals is an evidence of the cleavage of the ester bond and removal of CH_3–CH_2–CO part, which indicates the complete degradation of CP and formation of the Clobetasol. Fig. 6 represents the NMR spectrum of propionic acid; it shows signals corresponding to the aliphatic protons in CH_3 and CH_2 at 1.79 and 2.06 ppm, respectively, besides the corresponding signal to the carboxylic OH at 13.1 ppm.

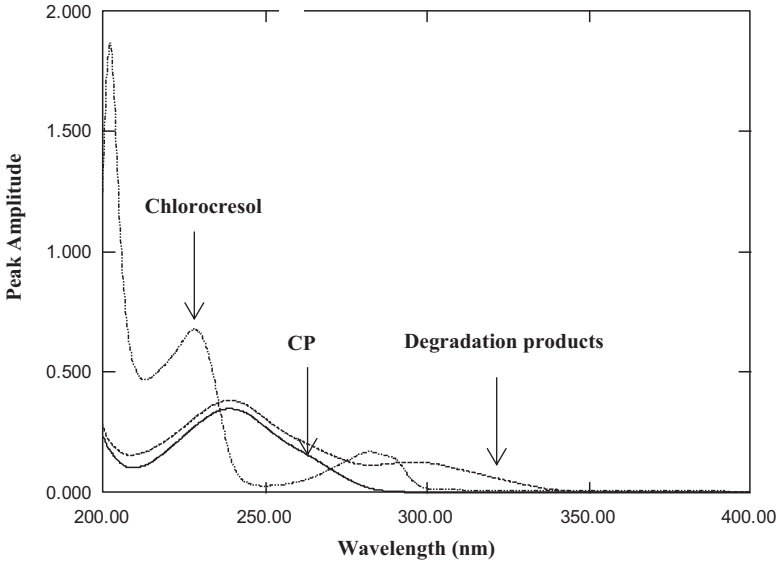


Figure 9 Zero order spectra of 10 μg mL^{-1} of Clobetasol propionate (—), its alkaline degradation products (– –) and Chlorocresol (–·–·–) using methanol as a blank.

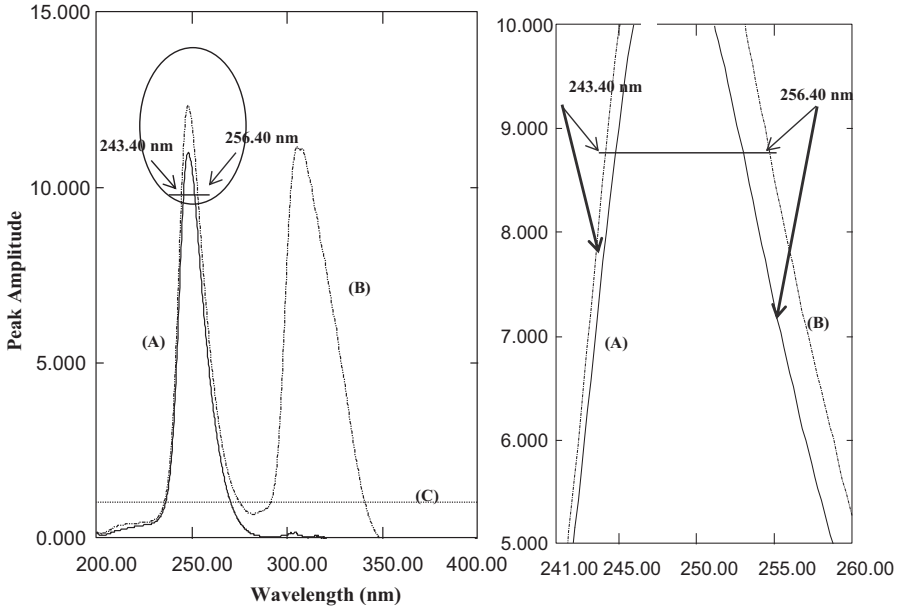


Figure 10 Ratio spectra of 10 μg mL^{-1} of Clobetasol propionate (—), its alkaline degradation products (–·–) and Chlorocresol (- - - -) using the spectrum of 10 μg mL^{-1} Chlorocresol in methanol as a divisor.

Table 2 Determination of Clobetasol propionate in laboratory prepared mixtures by the proposed methods.

Degradation products %	TLC-densitometric method			Degradation products %	Ratio difference method		
	Concentration (μg/band)		Recovery % of Clobetasol propionate		Concentration (μg mL^{-1})		Recovery % of Clobetasol propionate
	Degradation products	Clobetasol propionate			Degradation products	Clobetasol propionate	
10	0.05	0.45	101.33	2.5	0.25	9.75	97.23
20	0.10	0.40	100.00	10	1.00	9.00	99.56
30	0.15	0.35	100.57	20	2.00	8.00	99.00
40	0.20	0.30	99.33	30	3.00	7.00	99.71
50	0.25	0.25	100.00				
Mean ± SD			100.25% ± 0.75				98.88% ± 1.14

4.1. TLC-densitometric method

TLC-densitometry is an important technique in the field of separation and analysis of closely related compounds. This method offers good results regarding accuracy, precision and system suitability through scanning the chromatogram at the maximum peak of CP (λ_{max} 240 nm). Also, it allows quantification of a wide concentration range of CP. Good separation was obtained using ethyl acetate:hexane:ammonia (5:5:0.2, by volume) as a developing system as shown by the difference in the retention factor (R_f) values of CP and its hydrolytic degradation products (1 & 2) were R_f = 0.63, 0.30 and 0.56, respectively, Fig. 7.

The suitable developing system for CP was found to be ethyl acetate:hexane.[20] To optimize the chromatographic method different ratios were tried for separation e.g. (8:2, v/v), (7:3, v/v) and (6:4, v/v), where complete separation of CP and its degradation products were achieved using the ratio (1:1, v/v). Different scanning wavelengths were tried, where 240 nm offers the best result regarding sensitivity, peak symmetry and sharpness, Fig. 8. The optimum band width chosen was 6 mm and the inter-space between bands was 14 mm. Different slit dimensions were tried, where 5 mm × 0.2 mm proved to be the slit dimension of choice which provides the highest sensitivity.

System suitability parameters including resolution (R_s), peak symmetry, capacity factor (K') and selectivity factor (α) were calculated. The resolution was always above 1.5, the selectivity more than one and an accepted value for symmetry factor was obtained, Table 1. The linearity of CP was checked at the selected wavelength 240 nm in the concentration range of 0.1–0.5 μg/band. Calibration curve was constructed by plotting the integrated peak areas versus the concentrations of CP and the regression equation was calculated and found to be:

$$P = 6521.80\ C + 273.60 \quad r = 0.9992$$

where P is the integrated area under the peak for CP, C is the concentration of CP in μg/band and r is the correlation coefficient.

4.2. Spectrophotometric methods

Direct spectrophotometry failed to determine CP in the presence of its alkaline degradation products, due to severe overlapping of their spectra Fig. 9. When first derivative ratio method (^{1}DD) was applied as a stability indicating method using the degradation products as a divisor, it succeeded to determine CP in the presence of its alkaline degradation products only in the bulk pure powder of CP. Accurate and valid results were obtained, but upon applying the ^{1}DD method on the CP pharmaceutical formulation different results were obtained rather than expected. By more investigations, it was found that the presence of Chlorocresol as a preservative interferes with the method. Many trails were applied to the ^{1}DD method in order to determine CP in the presence of both its alkaline degradation products and its preservative Chlorocresol, but all trails failed due to severe overlapping of their spectra Fig. 9. This problem was solved by applying an alternative spectrophotometric method which is a ratio difference spectrophotometric method.[28]

Table 3 Determination of Clobetasol propionate in Dermovate® cream by the proposed methods and application of standard addition technique.

Pharmaceutical formulation		TLC-densitometric method	Ratio difference method
Dermovate® cream 0.05 g CP/100 g cream Batch No. 101072	Found %[a]	98.38 ± 2.15	101.30 ± 1.84
	Recovery of standard added % [a]	100.89 ± 1.92	98.40 ± 1.65

[a] Average of three determinations.

4.2.1. Ratio difference spectrophotometric method

Ratio difference method was developed for simultaneous determination of CP in the presence of both its alkaline degradation products and Chlorocresol (preservative) in CP bulk powder and pharmaceutical formulation.

The utility of Ratio difference data processing is to calculate the unknown concentration of a component of interest present in a mixture with an unwanted interfering component by calculating the peak amplitude difference between two wavelengths on the ratio spectra. This is directly proportional to the concentration of the component of interest, independent of the interfering components. The pre-requisite for the ratio difference method is the careful selection of the divisor concentration and the two such wavelengths where the interfering component shows same absorbance whereas the component

Table 4 Assay validation parameters of the proposed methods for the determination of Clobetasol propionate.

Parameters	TLC-densitometric method	Ratio difference method
Linearity		
Slope	6521.8	0.04
Intercept	273.6	0.05
Correlation coefficient (r)	0.9992	0.9996
Accuracy (mean ± SD)	99.60 ± 1.09	99.44 ± 1.60
Precision (% RSD)		
Repeatability*	±1.65	±1.15
Intermediate precision**	±0.89	±1.11
Specificity and selectivity	100.67 ± 1.09	98.88 ± 1.14
Range	0.10–0.50 μg/band	2.50–25.00 μg mL^{-1}
Robustness***	98.59 ± 1.43	–
LOD****	0.01 μg/band	0.58 μg mL^{-1}
LOQ****	0.04 μg/band	1.77 μg mL^{-1}

* The intraday (n = 9), average of three different concentrations repeated three times within the day.
** The interday (n = 9), average of three concentrations repeated three times in three successive days.
*** Robustness was determined by changing the developing system to ethyl acetate:hexane (27:23, v/v).
**** Limit of detection and quantitation are determined via calculations, LOD = (SD of the response/slope) × 3.3; LOQ = (SD of the response/slope) × 10.

Table 5 Statistical comparison of the results obtained by the proposed methods and the reported HPLC method for the determination of Clobetasol propionate in pure powder form.

	TLC-densitometric method	Ratio difference method	Reported method [24]*
Mean	99.6	99.44	99.54
SD	1.09	1.6	1.91
RSD%	1.09	1.61	1.92
n	9	9	9
Variance	1.19	2.56	3.65
F-value p = 0.05	3.07 (3.44)	1.43 (3.44)	–
Student's t-test	0.08 (2.12)	0.12 (2.12)	–

– The figures between parenthesis are the corresponding theoretical values of t and F at p = 0.05.
* HPLC method[24] using C_{18} column, methanol: water (68:32 v/v) as mobile phase, UV detection at 239 nm and a flow rate of 0.9 mL min^{-1}.

of interest shows significant difference in peak amplitude with concentration.[28]

The ratio difference method was found to be simple, accurate, economic and rapid. The interference of Chlorocresol was canceled by dividing both CP and degradation product spectra by the chosen concentration of Chlorocresol. After testing several divisors of Chlorocresol absorption spectra, best results were obtained using a spectrum of 10 μg mL^{-1}. Then two wavelengths on CP and its degradation products ratio spectra were carefully selected at which the peak amplitude difference of the degradation products is zero, as the peak amplitude is the same for the degradation products at those two wavelengths. The difference in peak amplitude at 243.40 and 256.40 nm was selected for CP, where the difference is zero for its degradation products, Fig. 10.

Linear relationship was obtained in the range of 2.5–25 μg mL^{-1} between the peak amplitude difference at 243.40 and 256.40 nm and the corresponding drug concentrations. The regression equation was computed and found to be:

$$\Delta P \cdot A_{243.40-256.40\ \mathrm{nm}} = 0.04\ C + 0.05 \quad r = 0.9996$$

where $\Delta P{\cdot}A$ is the peak amplitude difference between 243.40 and 256.40 nm, C is the concentration of CP in μg mL^{-1} and r is the correlation coefficient.

To assess the stability-indicating efficiency of the proposed methods, the degradation products of CP and Chlorocresol (preservative) were mixed with the intact drug in different ratios and analyzed by the proposed methods. Table 2 illustrates good selectivity in the determination of CP in the presence of its alkaline degradation products by preparing different mixtures containing 10–50% (for TLC method) and 2.5–30% of the degradation products (for the ratio difference method).

The suggested methods were successfully applied for the determination of CP in its pharmaceutical formulation, showing good percentage recoveries. The validity of the suggested methods was further assessed by applying the standard addition technique, Table 3. The suggested methods were validated according to the ICH guidelines[26] and the obtained results were accurate, precise and more sensitive in comparison with the other published work,[19] Table 4. The results obtained by the suggested methods were statistically compared to a reported HPLC method[24] and no significant difference regarding accuracy and precision was obtained, Table 5.

5. Conclusion

The present work was concerned with the determination of CP in the presence of its alkaline degradation products where simple, sensitive and rapid methods were described for the determination of CP in its pure form and pharmaceutical formulation according to the results obtained. The methods were validated according to ICH guidelines.

The presented TLC-densitometric method could provide highly selective quantitative stability indicating method for the analysis of CP in the presence of its alkaline degradation products. Different spectrophotometric methods were applied for the determination of CP including ^{1}DD method. Many trails were applied to the ^{1}DD method in order to determine CP in the presence of both its alkaline degradation products and its preservative Chlorocresol, but all trails failed due to severe overlapping of their spectra. Alternative ratio difference spectrophotometric method was developed. The proposed ratio difference method showed accurate and precise results and also provided the advantage of simultaneous determination of CP in the presence of both its alkaline degradation products and Chlorocresol as a preservative. Both TLC-

densitometric and ratio difference methods have the advantage of successfully determining CP in its available dosage form.

Conflict of interest

There is no conflict of interest.

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