Saudi Pharmaceutical Journal (2013) **21**, 411–421

King Saud University

Saudi Pharmaceutical Journal

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ORIGINAL ARTICLE

A comparative study of validated spectrophotometric and TLC- spectrodensitometric methods for the determination of sodium cromoglicate and fluorometholone in ophthalmic solution

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Received 8 October 2012; accepted 10 November 2012
Available online 17 November 2012

KEYWORDS

Absorptivity factor;
Absorption factor;
Mean centering;
TLC- spectrodensitometric;
Sodium cromoglicate;
Fluorometholone

Abstract The determination of sodium cromoglicate (SCG) and fluorometholone (FLU) in ophthalmic solution was developed by simple, sensitive and precise methods. Three spectrophotometric methods were applied: absorptivity factor (a-Factor method), absorption factor (AFM) and mean centering of ratio spectra (MCR). The linearity ranges of SCG were found to be (2.5–35 μg/mL) for (a-Factor method) and (MCR); while for (AFM), it was found to be (7.5–50 μg/mL). The linearity ranges of FLU were found to be (4–16 μg/mL) for (a-Factor method) and (AFM); while for (MCR), it was found to be (2–16 μg/mL). The mean percentage recoveries/RSD for SCG were found to be 100.31/0.90, 100.23/0.57 and 100.43/1.21; while for FLU, they were found to be 100.11/0.56, 99.97/0.35 and 99.94/0.88 using (a-Factor method), (AFM) and (MCR), respectively. A TLC-spectrodensitometric method was developed by separation of SCG and FLU on silica gel 60 F_{254} using chloroform:methanol:toluene:triethylamine in the ratio of (5:2:4:1 v/v/v/v) as developing system, followed by spectrodensitometric measurement of the bands at 241 nm. The linearity ranges and the mean percentage recoveries/RSD were found to be (0.4–4.4 μg/band), 100.24/1.44 and (0.2–1.6 μg/band), 99.95/1.50 for SCG and FLU, respectively. A comparative study was conducted between the proposed methods to discuss the advantage of each method. The suggested methods were validated in compliance with the ICH guidelines and were successfully applied for the deter-

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.jsps.2012.11.001

mination of SCG and FLU in their laboratory prepared mixtures and commercial ophthalmic solution in the presence of benzalkonium chloride as a preservative. These methods could be an alternative to different HPLC techniques in quality control laboratories lacking the required facilities for those expensive techniques.

1. Introduction

Sodium cromoglicate (SCG) [disodium 4,4′-dioxo-5,5′-(2-hydroxytrimethylenedioxy)di(4*H*-chromene-2-carboxylate)] is a mast cell stabilizer that inhibits the release of histamine and other inflammatory mediators from sensitized mast cells. Fluorometholone (FLU) [9α-fluoro-11b,17α-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione] is a corticosteroid employed for its glucocorticoid activity. Both drugs are formulated together in the form of ophthalmic solution for treatment of allergic conjunctivitis and vernal keratoconjunctivitis (Sweetman, 2005). The chemical structures of both drugs (British Pharmacopoeia, 2009) were shown in Fig. 1.

A survey of the literature revealed that the methods reported for the determination of SCG are UV spectrophotometry (Hassib et al., 2011; Tillman and Whymark, 1971), HPLC (Ali et al., 2008; Ozoux et al., 2001), TLC (Kocić-Pesić et al., 1992) and capillary electrophoresis (Helle et al., 2008). The methods reported for FLU are UV spectrophotometry (Vladimirov et al., 1996; Altuntas et al., 2000) and HPLC (Korpi-Steiner et al., 2010; Croesa et al., 2009; O'keeffe et al., 2003). No methods have been reported for the analysis of the binary mixture of the two drugs. This work aims to present simple, accurate and precise spectrophotometric and TLC- spectrodensitometric methods for the simultaneous determination of SCG and FLU.

1.1. Theory of the novel absorptivity factor spectrophotometric method (a-Factor method)

Samir et al. (2012) introduced the novel absorptivity factor spectrophotometric method. It depends on applying a simple mathematic equation to calculate the concentration of both components in a binary mixture where there is a large difference in the absorptivity between both components in a mixture, so that no isoabsorptive point occurs. In this case simultaneous determination of each drug in the presence of the other cannot be carried out by applying the isoabsorptive point technique.

For a mixture of two drugs X and Y, the concentration of Y can be determined by using any of the well-established spectrophotometric methods, while X can only be determined using the absorptivity factor method. This method depends on the calculation of the absorptivity factor which is the ratio between the two absorptivities (a_x, a_Y) at an intersection point with the same absorbance value. This point is called the absorptivity factor point (λ_F). This is summarized as follows:

$$A_x = A_y$$

$$a_x b_x C_x = a_y b_Y C_Y \quad (\text{where } b_x = b_Y = 1cm)$$

$$a_x C_x = a_y C_Y$$

$$a_x/a_y = C_y/C_x$$

$$a_x/a_y = F$$

$$a_x = F a_y$$

where, F is the absorptivity factor and a_x and a_Y are the absorptivities of X and Y respectively.

For a mixture of X and Y the total absorbance of X and Y at absorptivity factor point λ_F in UV can be expressed as follows:

$$A_m = A_x + A_y$$

$$A_m = a_x b_x C_x + a_y b_Y C_Y \quad (\text{where } b_{x=} b_Y = 1cm)$$

$$A_m = a_x C_x + a_y C_Y$$

where

A_X, A_y and A_m are the UV absorbance of X, Y and their mixture at λ_F respectively.

C_x and C_Y are the concentrations of X and Y respectively. a_x and a_Y are the absorptivities of X and Y at λ_F respectively.

a_x is substituted by $F\,a_y$.

$$A_m = a_Y F C_x + a_Y C_Y$$

$$A_m = a_Y (\mathrm{F}C_x + C_Y)$$

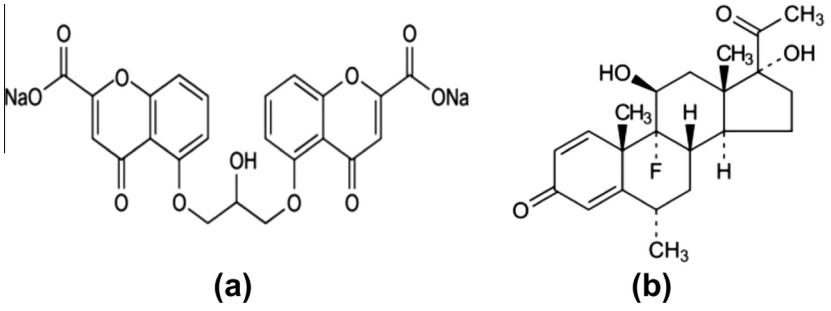

Figure 1 The chemical structures of (a) sodium cromoglicate (SCG) and (b) fluorometholone (FLU).

So, the concentration of the mixture that represents ($FC_x + C_Y$) can be calculated by using a regression equation representing the linear relationship between the absorbance of Y and its corresponding concentration at the absorptivity factor point. Thus, if the concentration of Y can be determined separately by any other method such as direct spectrophotometry, derivative or derivative ratio, then the concentration of X can be determined after subtraction of concentration Y and multiplication by the inverse of F or vice versa.

$$C_X = (FC_x + C_Y) - C_Y = FC_x$$

$$C_x = FC_x X\ 1/F$$

Or

$$C_Y = (FC_x + C_Y) - C_x XF$$

2. Experimental

2.1. Apparatus and software

Shimadzu - UV 1800 double beam UV–visible spectrophotometer (Japan) with matched 1 cm quartz cells at 200–800 nm range was used for all absorbance measurements. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software. For the mean centering for ratio spectra method, Matlab® Version 7.9 was used.

The TLC- spectrodensitometric system: Camag TLC scanner 3 S/N 130319 operated with winCATS software, Linomat 5 autosampler (Switzerland), Camag microsyringe (100 μL) and TLC aluminum sheet (20 × 20 cm) precoated with silica gel 60 F_{254} (Merck KgaA, Darmstad, Germany) were used.

2.2. Chemicals and reagents

2.2.1. Pure samples

Sodium cromoglycate (SCG) was kindly supplied by Sigma Pharmaceutical Industries Limited, Al-Monofeya, Egypt. While fluorometholone (FLU) was kindly supplied by Egypt Egyptian International Pharmaceutical Industries Co., (EIPICO), Cairo, Egypt. Their purity was found to be 100.80 ± 0.74 and 100.12 ± 0.52 for SCG and FLU respectively by the official methods (British Pharmacopoeia, 2009).

2.2.2. Market sample

Fluca® eye drops, labeled to contain 20 mg of SCG and 1 mg of FLU per 1 mL (batch no. LM036), were manufactured by Jamjoompharma, Kingdom of Saudi Arabia.

2.2.3. Solvents

Spectroscopic analytical grade of methanol, chloroform, and triethylamine, supplied from (S.d.fine-chem limited- Mumbai); analytical grade of Toluene supplied from (Adwic- El Nasr pharmaceutical Chemicals Co., Egypt) and distilled water were used.

2.3. Standard solutions

Stock solutions were prepared in a solvent mixture of methanol:water (50:50 v/v), of concentration 1 mg/mL and 0.5 mg/mL, respectively; and stored in dark bottles at 4 °C.

Working solutions were freshly prepared by dilution from the stock solutions with solvent mixture to get 50 μg/mL and 20 μg/mL (for spectrophotometric methods) and 800 and 400 μg/mL (for TLC- spectrodensitometric method) of SCG and FLU respectively.

2.4. Procedure

2.4.1. For spectrophotometric methods

2.4.1.1. Linearity. 2.4.1.1.1. Linearity of SCG. Aliquots (0.5–8 mL) of SCG working solution (50 μg/mL) were transferred into a series of 10 mL volumetric flasks, and the volume was completed with solvent mixture. The absorption spectra were measured for the prepared solutions at (200–400 nm). Calibration curves were constructed relating the D_0 absorbance at 227.8 and 325 nm to the corresponding concentration against solvent mixture as a blank and the regression equation was computed. The absorption factor for SCG was calculated by dividing the absorbance at λ_1 (241 nm)/λ_2 (325 nm). The first derivative D^1 was calculated for the obtained spectra. Calibration curve was constructed relating the D^1 amplitude at 241.5 nm to the corresponding concentration and the regression equation was computed. The zero order spectra of the prepared solutions were divided by the spectrum of 8 μg/mL FLU. The ratio spectra were exported to Matlab to perform mean centering with respect to wavelength (200–300 nm) to obtain the mean centered ratio spectra (MC). Calibration curve was constructed relating the peak amplitude of MC at 252.2 nm to the corresponding concentrations and the regression equation was computed.

2.4.1.1.2. Linearity of FLU. Aliquots (1.0–8.0 mL) of FLU working solution (20 μg/mL) were transferred into a series of 10 mL volumetric flasks, and the volume was completed with solvent mixture. The absorption spectra were measured for the prepared solutions at (200–400 nm). Calibration curves were constructed relating the D_0 absorbance at 241 and 227.8 nm to the corresponding concentration against solvent mixture as a blank and the regression equation was computed. The zero order spectra of the prepared solutions were divided by the spectrum of 10 μg/mL SCG.

The ratio spectra were exported to Matlab to perform mean centering with respect to wavelength (200–350 nm) to obtain the mean centered ratio spectra (MC). Calibration curve was constructed relating the maximum peak amplitude of MC at 252.2 nm to the corresponding concentrations and the regression equation was computed.

2.4.1.2. Application to laboratory prepared mixtures. Into two series of 10 mL volumetric flasks (A and B), accurate aliquots of SCG and FLU were transferred from their working solutions (50 and 20 μg/mL), respectively, to prepare mixtures containing different ratios of the two drugs. Each flask in the second series (B) was spiked with 40 μg of FLU from its working solution (2 mL). Complete both series to volume with the solvent mixture. Record and store the spectra of the prepared solutions of both series from 200 to 400 nm. SCG was determined using series A, FLU was determined using series B. Proceed as detailed under linearity for both drugs. The concentration of each drug was calculated using the corresponding regression equation. The claimed concentration of FLU in each mixture was calculated after subtraction of the

added concentration (FLU solution 4 μg/mL analyzed by using the same procedure).

2.4.1.3. Application to pharmaceutical preparation. One milliliter was accurately transferred from Fluca® eye drops to a 100 mL volumetric flask and diluted to the mark with solvent mixture to get 200 μg/mL of SCG and 10 μg/mL of FLU; the prepared solution was filtered through a 0.45 μm Millipore syringe membrane filter. One milliliter of the prepared solution was transferred into two separate 10 mL volumetric flasks. Complete the first flask to volume with solvent mixture to obtain solution with a final concentration 20 μg/mL of SCG and 1 μg/mL of FLU, while the second flask was spiked with 40 μg of FLU from its working solution (2 mL) and complete to volume with solvent mixture. SCG was determined from the first flask and FLU was determined from the second flask. Proceed as detailed under linearity for both drugs. The concentration of each drug was calculated using the corresponding regression equation. The claimed concentration of FLU in the solution was calculated after subtraction of the added concentration (FLU solution 4 μg/mL analyzed by using the same procedure). When carrying out the standard addition technique, different known concentrations of pure standard SCG and FLU were added to the pharmaceutical dosage form before proceeding in the previously mentioned methods.

2.4.2. For TLC-spectrodensitometric method

2.4.2.1. Chromatographic conditions. TLC aluminum sheets 20×10 cm precoated with 0.25 mm silica gel 60 F_{254} were used. The samples were applied as bands (bandwidth: 6 mm, bands were spaced 1 cm apart from each other and 1.5 cm from the bottom edge of the plate). The developing system was chloroform:methanol:toluene:triethylamine (5:2:4:1 v/v/v/v). Linear ascending development was done in a chromatographic tank previously saturated with the developing system for 1 h at room temperature to a distance of approximately 8 cm from the lower edge. The developed plates were air dried and scanned at 241 nm. The detection was done using Camage TLC scanner 3 operated in the absorbance mode; with deuterium lamp as a source of radiation; the slit dimension was kept at $3\ \text{mm} \times 0.45\ \text{mm}$ and 20 mm/s scanning speed was employed.

2.4.2.2. System suitability. Parameters including resolution (Rs) and peak symmetry were calculated and compared to reference value (United States Pharmacopoeia Commission (2004).

2.4.2.3. Linearity. Aliquot volumes (0.5–5.5 mL) of SCG and (0.5–4 mL) of FLU were separately transferred from their working solutions into 10 mL volumetric flasks and diluted to volume with solvent mixture. Ten microliters of each solution was applied to a TLC plate using a 100 μL syringe. The chromatographic conditions were applied and the chromatograms were recorded. The calibration curves were plotted between the recorded peak area $\times 10^{-2}$ and the corresponding concentrations, from which the regression equations were calculated. The calibration curves were made from the average of three experiments.

2.4.2.4. Assay of laboratory-prepared mixtures. Different aliquot volumes of both drugs were accurately transferred from their working solutions and mixed to prepare solutions of different ratios. Ten microliters of each solution was applied to a TLC plate using a 100 μL syringe. The chromatographic conditions were adopted for each laboratory- prepared mixture and the concentrations for each drug were calculated from the regression equations. Each concentration was conducted from the average of three experiments.

2.4.2.5. Application to pharmaceutical preparation. Five milliliters of Fluca® eye drops was transferred into 25 mL volumetric flask, the volume was completed with solvent mixture to get 4000 μg/mL of SCG and 200 μg/mL of FLU. The prepared solution was filtered through 0.45 μm Millipore syringe membrane filter and an appropriate dilution was made from the same solvent to prepare a solution of 400 μg/mL SCG and 20 μg/mL FLU. Ten microliters of the solution was applied to a TLC plate using a 100 μL syringe. The chromatographic conditions were applied and the chromatograms were recorded. Six replicates were done .The concentrations of SCG and FLU were calculated from their corresponding regression equations. The standard addition technique was applied by adding different known concentrations of pure standard drugs to the pharmaceutical formulation before proceeding in the previously mentioned method.

3. Results and discussion

The aim of this work was to develop simple, sensitive, selective and precise spectrophotometric and TLC-spectrodensitometric methods for simultaneous estimation of SCG and FLU in their pure form and ophthalmic solution.

3.1. For spectrophotometric methods

Two major problems raised upon the analysis of this binary mixture which were: the overlapped spectra with a large difference between the absorptivities of the two drugs; and the ratio of the drugs in the dosage form SCG:FLU (20:1) which make it difficult to determine FLU accurately. On the analysis of mixtures of compounds of largely variable absorptivities, there is a need to use higher concentrations of the component mixture solution to analyze those with low absorptivity which may lead to a deviation from Beer's law due to the electrostatic attraction between ions (Harvey, 2000). To facilitate the determination of the minor component (FLU), there is a need to increase its concentration in the binary mixture using sample enrichment technique. This is done adding fixed amounts of standard FLU to each experiment then subtract its concentration before calculating the claimed concentration of the drug. This technique has been used to solve the same problem for analyzing other drug mixtures (Lotfy, 2006; Lotfy and Hagazy, 2012). The UV absorption spectra (D_0) of SCG and FLU are shown in Fig. 2, where FLU could not be resolved by direct derivative spectrophotometry. So, different spectral manipulating techniques have been applied. All spectral measurements were done without interference of benzalkonium chloride which did not show any absorption and its contribution to the absorption of the mixture above 220 nm was considered to be negligible at a concentration up to100 μg/mL, so the ternary mixture in the range (220–400 nm) acts as a binary mixture of FLU and SCG.

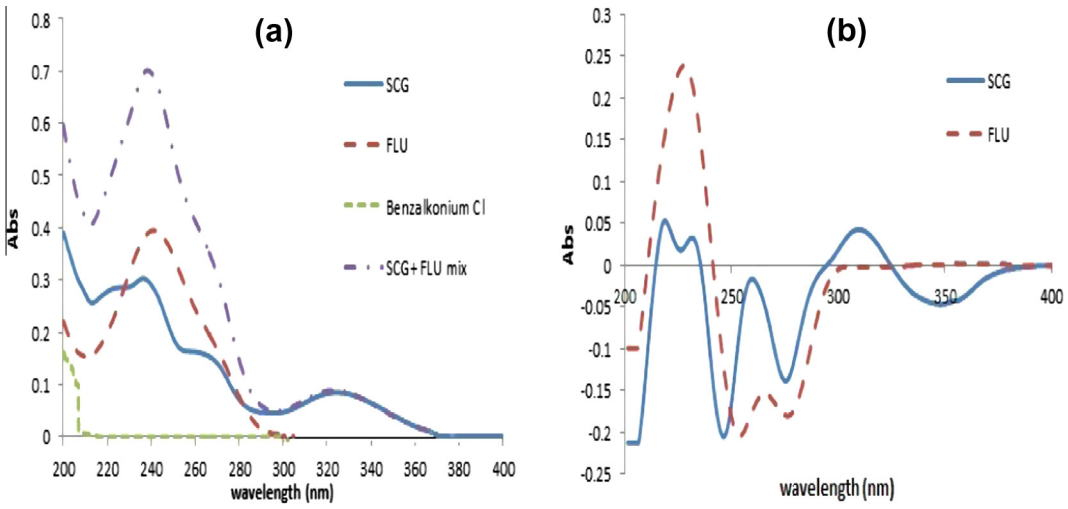

Figure 2 (a) UV spectra of 5 μg/mL of SCG, 10 μg/mL of FLU and 10 μg/mL of benzalkonium chloride, (b) 1st derivative spectra D^1 of 5 μg/mL of SCG and 10 μg/mL of FLU.

3.1.1. a-Factor method

This method is considered as a modification of isoabsorptive technique, but in the isoabsorptive technique the spectra of the same concentrations of the two studied drugs should cross at a point called isoabsorptive point at which they have equal absorptivities, while in absorptivity factor method the crossing point did not occur at equal concentration so the crossing point is obtained between different concentrations of the two drugs. At this point the absorptivities of the two drugs are not equal but they are equal to the inverse of the ratio of the used concentrations.

The UV absorption spectra (D_0) of SCG and FLU are shown in Fig. 2, showing two crossing absorptivity factor points (λ_F) at 227.8 and 282 nm. Both points were tested for the determination of mixture, but the later one (282 nm) showed a bad linearity correlation (0.9955) for both drugs due to their low absorptivities at this wavelength, subsequently bad recoveries were obtained for the mixtures. So the first point 227.8 nm (linearity correlation = 0.9999) was chosen to calculate the absorptivity factor where the absorptivity of SCG is double that of FLU ($a_{SCG}/a_{FLU} = F = 2$). The concentration of the mixture that represents ($C_{FLU} + 2C_{SCG}$) can be calculated by using a regression equation representing the linear relationship between the absorbance of FLU and its corresponding concentration at the absorptivity factor points λ_F (227.8 nm). Thus, the concentration of SCG (C_{SCG}) in the mixtures can be determined separately by using its D_0 at 325 nm in a concentration range of (7.5–40 μg/mL); or by using the first derivative spectra $D^1$of SCG to solve its overlapping with FLU, Fig. 2, measured at 241.5 nm in a concentration range with higher sensitivity (2.5–35 μg/mL) due to its higher absorptivity at this wavelength. Then the concentration of FLU (C_{FLU}) can be calculated as follows:

$$A_{mix} = a_{FLU}(C_{FLU} + 2C_{SCG}) at \lambda_F$$

$$C_{FLU} = (C_{FLU} + 2C_{SCG}) - (2XC_{SCG}).$$

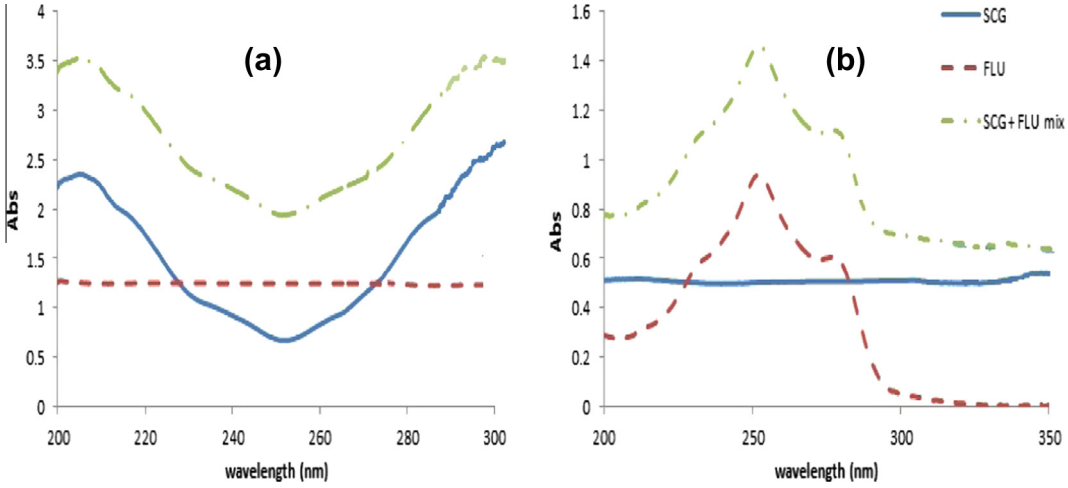

Figure 3 Ratio spectra of SCG (5 μg/mL), FLU (10 μg/mL) and mixture of 5 μg/mL of SCG and 10 μg/mL of FLU (a) using 8 μg/mL of FLU as a divisor, (b) using 10 μg/mL of SCG as a divisor.

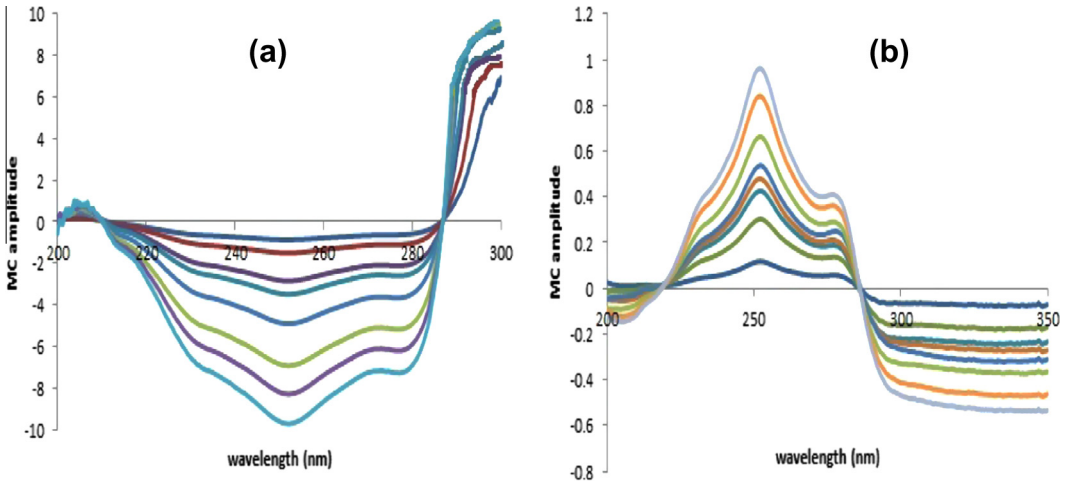

Figure 4 Mean centered ratio spectra of (a) SCG (2.5–35 μg/mL) using 8 μg/mL FLU as a divisor and (b) FLU (2–16 μg/mL) using 10 μg/mL SCG as a divisor.

where A_{mix} the absorbance of the mixture (C_{FLU} + $2C_{SCG}$) at λ_F (227.8 nm), C_{FLU} and C_{SCG} are the concentrations of FLU and SCG, respectively.

3.1.2. AFM

This method (Patel et al., 2007; Prajapati et al., 2011) describes the analysis of a binary mixture where the two components X and Y have overlapped spectra. Y shows interference at $_{max}$ of X ($_1$), while X shows no interference with Y at another wavelength ($_2$). As shown in Fig. 2, the absorption spectra of SCG and FLU show severe overlap in the wavelength region of 200–300 nm. So, the absorption spectra of the standard solutions of the SCG with different concentrations were recorded in the wavelength range of 200–400 nm, and the average value of absorption factor (abs 241 nm/abs 325 nm) was found to be 3.355. Since the absorbance of the mixture (SCG + FLU) at 325 nm is equal to that of pure SCG due to lack of contribution of FLU at this wavelength, the absorption of FLU could be calculated using the following equation:

$$\text{Absorption of FLU at} 241nm = abs_{241}(\text{SCG} + \text{FLU}) - \frac{abc1}{abs2} \times abs_{325}(\text{SCG} + \text{FLU})$$

where; $\frac{abc1}{abs2}$ is the absorption factor of pure SCG (abs 241 nm/abs 325 nm = 3.355), abs $_{241}$ (SCG + FLU) is the absorbance of the mixture at 241 nm, but abs $_{325}$ (SCG + FLU) = abs $_{325}$ SCG only at 325 nm.

The concentrations of SCG and FLU were calculated from the corresponding regression equation obtained by plotting the absorption values of the zero order spectra, at 325 and 241 nm, against the corresponding concentrations, respectively.

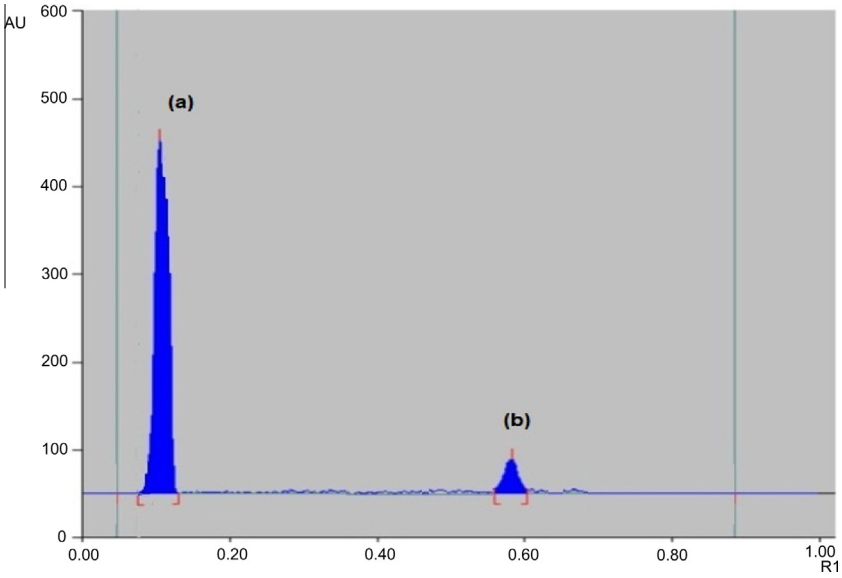

Figure 5 2D TLC chromatogram of (a) 4 μg/band of SCG and (b) 0.2 μg/band FLU using chloroform:methanol:toluene:triethylamine in ratio of (5:2:4:1 v/v/v/v) as developing system.

Table 1 Statistical analysis of parameters required for system suitability of TLC-spectrodensitometric method.

Parameter	OFX	DXM	Reference value Afkhami and Bahram (2004)
R_f value	0.1 ± 0.01	0.58 ± 0.02	
T (tailing factor)	0.83	1.01	$T < 2$ $T = 1$ for symmetric peak
Rs (experimental resolution)	10		Rs > 2

3.1.3. MCR

This is a well-established spectrophotometric method (Afkhami and Bahram, 2004; Zarei et al., 2009; Darwish et al., 2011) where the constant obtained in the ratio spectra of a mixture is removed by mean centering (MC). A linear relation is obtained for the (MC) amplitude of one component against its concentration. This method eliminates derivative steps and therefore signal-to-noise ratio is enhanced.

The ratio spectra for both drugs were obtained by testing different concentrations of the divisors (5, 10 and 15 μg/mL of SCG) and (4, 8 and 12 μg/mL of FLU) but the concentrations 10 μg/mL of SCG and 8 μg/mL of FLU gave minimum noise in ratio spectra and maximum sensitivity as shown in Fig. 3. The ratio spectra were mean centered in the range (200–300 nm) for SCG and (200–350 nm) for FLU, Fig. 4; the remaining spectra were eliminated as it affected the linearity of the MC curves. The concentration of both drugs was calculated by using the regression equation representing the linear relationship between (MC) at 252.2 nm for SCG and FLU.

3.2. For TLC-spectrodensitometric method

This method offers a simple way for quantification directly on TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing to a standard curve from reference materials chromatographed simultaneously under the same condition (Wagieh et al., 2010; Kasaye et al., 2010).

To optimize the method conditions, it was necessary to test the effect of different variables. In order to separate the two drugs from each other, several ratios of different developing systems were checked. Increasing the ratio of chloroform slightly affected the separation of SCG but it affected the migration of FLU to the solvent front. Methanol caused an increase in Rf of both drugs but SCG bands showed tailing and FLU bands migrated to the solvent front. Toluene affected the migration of FLU only to higher Rf but caused tailed peaks, while triethylamine was necessary to reduce the tailing of both peaks. Finally it was found that the best separation of SCG and FLU was obtained by applying the developing system using chloroform:methanol:toluene:triethylamine (5:2:4:1 v/v/v/v). Due to the great difference in polarities between the two drugs; SCG was separated at Rf = 0.1 ± 0.01 while FLU was separated at Rf = 0.58 ± 0.02. Different scanning wavelengths were tried (241, 236.3 and 325 nm); on using 241 nm, the separated peaks were more sharp and symmetrical with minimum noise, as shown in Fig. 5.

The parameters of system suitability of the TLC-spectrodensitometric are listed in Table 1. The corresponding concentration ranges, calibration equations and other statistical parameters for the proposed methods were listed in Table 2. The selectivity of the proposed procedures was assessed by the analysis of laboratory prepared mixtures containing different ratios of the two drugs, where satisfactory results were obtained over the calibration ranges as shown in Table 3. The proposed procedures were also applied for the determination

Table 2 Assay parameters and method validation obtained by applying the proposed methods.

Parameters	a-Factor method		AFM		MCR		TLC- method	
	SCG	FLU	SCG	FLU	SCG	FLU	SCG	FLU
Wavelength (in nm)	D^1 at 241.5	D_0 at 227.8	D_0 at 325	D_0 at 241	MC at 252.2	MC at 252.2	241	241
Calibration range[a]	2.5–35	4–16	7.5–50	4–16	2.5–35	2–16	0.4–4.4	0.2–1.6
LOD	0.426	0.105	0.189	0.089	0.343	0.163	0.107	0.034
LOQ	1.290	0.317	0.574	0.269	1.038	0.494	0.323	0.103
Slope	−0.0279	0.0283	0.0167	0.0397	−0.2733	0.0601	0.8206	1.7669
Intercept	−0.0067	0.0033	0.0011	0.0001	−0.1207	−0.0002	1.562	0.6891
Mean %[b]	100.31	100.11	100.23	99.97	100.43	99.94	100.24	99.95
RSD %	0.90	0.56	0.57	0.35	1.21	0.88	1.44	1.50
Accuracy[b]	100.73/0.97	101.2/0.80	100.11/0.20	100.9/0.92	100.63/0.91	100.24/1.23	99.02/0.72	100.60/0.75
Intra-day precision[c]	100.57/0.61	100.2/0.39	99.90/0.50	100.1/0.45	100.37/0.71	99.81/0.63	100.08/0.70	100.25/0.65
Inter-day precision[c]	100.21/0.46	100.2/0.46	100.37/0.40	100.02/0.55	100.11/0.79	100.10/0.59	100.04/0.64	100.12/0.23
Robustness	99.47/0.93	99.75/1.41	99.58/1.13	100.38/1.09	99.54/0.68	99.65/0.53	100.64/0.63	99.63/1.45
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999	0.9998	0.9999	0.9997	0.9997

LOD, limit of detection, LOQ, limit of quantitation; in μg/mL for spectrophotometric methods; in μg/band for TLC method.
D_0, zero order spectra; D^1, 1st derivative spectra; MC, mean centered spectra.
[a] Calibration points: $n = 8$ for spectrophotometric methods, $n = 7$ for TLC method, Concentration in μg/mL for spectrophotometric methods, μg/band for TLC method.
[b] Average of three experiments.
[c] Mean percentage recoveries/RSD of three samples of SCG and FLU.

Table 3 Determination of SCG and FLU in laboratory prepared mixtures by applying the proposed methods.

SCG:FLU in (μg/mL)[a] in (μg/band)[b]	a-Factor method		AFM		MCR		TLC	
	SCG[c]	FLU[a]	SCG	FLU[a]	SCG	FLU[a]	SCG[b]	FLU[b]
				Recovery % [d]				
10:10	100.12	99.62	99.34	98.64	100.83	100.00	98.33	101.67
10:5	99.74	98.40	99.94	100.27	98.38	102.23	99.38	101.25
20:4	102.93	101.00	101.47	99.87	99.22	99.67	99.75	100.00
20:2	101.68	99.76	100.87	100.72	101.07	98.96	99.50	102.50
20:1[e]	100.96	101.59	100.57	98.37	99.86	100.01	100.25	100.00
Mean ±SD	101.08 ±1.28	100.07 ±1.25	100.44 ±0.82	99.58 ±1.02	99.87 ±1.12	100.18 ±1.23	99.44 ±0.70	101.08 ±1.09

[a] Concentration of SCG and FLU is calculated in (μg/mL) after subtraction of spiked FLU concentration (4 μg/mL).
[b] Concentration of SCG and FLU is calculated in (μg/band).
[c] Using D^1 method at 241.5 nm.
[d] Average of three experiments.
[e] Ratio present in Fluca® eye drops.

of SCG and FLU in Fluca® eye drops; and were compared to the manufacturer HPLC method (Organization for Drug Control and - Quality Control Department, XXXX). The validity of the proposed procedures is further assessed by applying the standard addition technique showing no excipients interference. The results obtained were shown in Table 4.

4. Methods validation

Method validation was performed according to ICH guidelines (ICH, 2005) as follows:

4.1. Range and linearity

The linearity of the proposed methods was evaluated by processing the different calibration curves on three different days. The calibration curves were constructed within concentration ranges that were selected on the basis of the anticipated drugs concentration during the assay of the dosage form. The ranges for FLU were (2–16 μg/mL) using MCR, while for the a-factor method and AFM the ranges were found to be (4–16 μg/mL). This is contributed to the enhancement of the signal to noise ratio upon using MCR. Using AFM, SCG should be measured at its D_0 at 325 nm in a concentration range of (7.5–40 μg/mL), unlike the a-Factor method where we could utilize the $D^1$of SCG at 241.5 nm in a concentration range of (2.5–35 μg/mL) to increase the sensitivity of SCG determination. The corresponding concentration ranges, calibration equations and other statistical parameters for the proposed methods are listed in Table 2.

4.2. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated respectively, for both drugs using the proposed methods with a ratio of 3.3 and 10 standard deviations of the blank and the slope of the calibration line, Table 2.

Table 4 Application of standard addition technique to the analysis of SCG and FLU in Fluca® eye drops (batch no. LM036) by applying the proposed methods.

Methods	SCG			FLU		
	Found[a] in μg/mL [b] μg/band [c]	Found recovery % ± S.D	Pure added[d] recovery % ± S.D	Found[a] in μg/mL [b] μg/band [c]	Found recovery % ± S.D	Pure added[d] recovery % ± S.D
a-Factor method[b,e]	101.59 ± 0.48	100.24 ± 0.21	0.994	99.44 ± 0.81	99.93 ± 0.24	
AFM[b]	20.318	101.59 ± 0.39	100.39 ± 0.26	0.998	99.84 ± 0.52	100.14 ± 0.12
MCR[b]	20.301	101.50 ± 0.99	100.02 ± 0.07	0.994	99.38 ± 0.60	100.08 ± 0.46
TLC[c]	4.039	100.97 ± 0.27	100.60 ± 0.35	0.199	99.76 ± 0.44	100.26 ± 0.54
Manufacturer HPLC method Zarei et al. (2009)[b,f]	20.25	101.26 ± 0.52		0.993	99.31 ± 0.74	

[a] Average of six experiments.
[b] SCG claimed to be 20 μg/mL and FLU to be 1 μg/mL (after subtraction of spiked FLU concentration 4 μg/mL).
[c] SCG claimed to be 4 μg/band and FLU to be 0.2 μg/band.
[d] Average of three experiments (SCG and FLU added equivalent to 2, 4, 6 μg/mL, for TLC:0.4, 0.5, 0.6 μg/band).
[e] Using D^1 method at 241.5 nm.
[f] Manufacturer HPLC method using RP C18 and mobile phase: 4.243 g of tetrabutyl ammonium hydrogen sulfate + 5 ml of triethylamine in 1 L of water, pH 2.1 adjusted with o-H_3PO_4, flow rate 1 ml/min, detection at 254 nm.

Table 5 Statistical comparison between the results obtained by the proposed method and the official BP methods British Pharmacopoeia (2009) for the determination of SCG and FLU in pure powder form.

Items	SCG					FLU				
	a-Factor method [b]	AFM	MCR	TLC	Official method [a] British Pharmacopoeia (2009)	a-Factor method	AFM	MCR	TLC	Official method [a] British Pharmacopoeia (2009)
Mean %	100.28	100.23	100.43	100.24	100.80	100.11	99.97	99.94	100.23	100.12
RSD	0.78	0.57	1.21	1.74	0.74	0.56	0.35	0.88	0.57	0.52
SEM	0.28	0.20	0.46	0.66	0.33	0.20	0.13	0.31	0.36	0.23
n	8	8	8	7	5	8	8	8	7	5
Student's *t*-test (2.201)[c] (2.228)[d]	1.203	1.570	0.612	0.670		0.032	0.617	0.393	0.245	
F value (6.094)[c] (6.163)[d]	1.108	1.719	2.669	5.508		1.176	2.137	2.923	3.392	

SEM, standard error of mean.
[a] BP method for SCG is potentiometric titration method, while for FLU is HPLC method.
[b] Using D^1 method at 241.5 nm.
[c] Figures between parentheses represent the corresponding tabulated values of t and F at $P = 0.05$ at 11 degree of freedom.
[d] Figures between parentheses represent the corresponding tabulated values of t and F at $P = 0.05$ at 10 degree of freedom.

4.3. Accuracy

To study the accuracy of the proposed methods, procedures under study of linearity, for both drugs using the proposed methods, were repeated three times for the determination of different concentrations of SCG and FLU within the linearity range. The accuracy expressed as mean percentage recoveries and relative standard deviation (RSD) was shown in Table 2. The interference of excipients in the pharmaceutical formulations was studied by applying standard addition method to the pharmaceutical formulation. Good accuracy proved that the excipients in pharmaceutical formulation did not interfere in the analysis of these compounds in the pharmaceutical formulation as shown in Table 4.

4.4. Precision

The inter-day and intra-day precisions of the proposed methods were determined by the analysis of three different concentrations of SCG and FLU, within the linearity range, by three replicate analyses of three pure samples of both drugs on a single day and on three consecutive days, respectively. The results expressed as mean percentage recoveries and RSD are illustrated in Table 2.

4.5. Selectivity

Selectivity was ascertained by analyzing different mixtures containing both drugs in different ratios within the linearity range. Satisfactory results were shown in Table 3.

4.6. Robustness

For the spectrophotometric methods, testing for robustness involved performing the spectral measurements at ± 1 nm for three different concentrations of SCG and FLU. The robustness was calculated as mean percentage recoveries and relative standard deviation as shown in Table 2, where the a-Factor method showed the highest value of RSD. This could be justified by the critical measurement of absorbance at absorptivity factor point (λ_F) at 227.8 nm as any minor change in the selected wavelength will affect the results and subsequently shows poor robustness. For the TLC-spectrodensitometric methods, the analysis of samples was done under a variety of experimental conditions, such as small changes in proportions of different components by up to $\pm 0.5\%$ mainly of the organic parts of the developing systems. The effect on Rf values and peak parameters was studied. The methods proved to be

Table 6 Results of ANOVA (single factor) for comparison of the proposed methods for the determination of SCG and FLU in pharmaceutical dosage form.

Source of variation		Degree of freedom	Sum of squares	Mean square	*F* value [a]	*P* value [a]
SCG	Between columns	3	1.592	0.5306	1.455	0.2568
	Within columns	20	7.294	0.3647		
	Total	23	8.885			
FLU	Between columns	3	0.9224	0.3075	0.8281	0.4939
	Within columns	20	7.427	0.3713		
	Total	23	8.349			

[a] There was no significant difference between the methods using one-way ANOVA at $P < 0.05$.

robust and the percentage recoveries and RSD were calculated, Table 2.

5. Statistical analysis

Table 5 showed statistical comparison of the results obtained by the proposed methods and official methods (British Pharmacopoeia, 2009). The calculated t and F values were less than the theoretical ones indicating that there was no significant difference between the proposed and the official methods with respect to accuracy and precision. One-way ANOVA was applied for the purpose of comparison of developed methods; Table 6 shows that there was no significant difference between them.

6. Conclusion

The main advantage of the absorptivity factor method (a-Factor method) and absorption factor method (AFM) over the mean centering of ratio spectra method (MCR) is the application of simple mathematic equation to calculate the concentration of both components in binary mixture with different absorptivities either at the same wavelength (absorptivity factor method) or at different wavelength (absorption factor method) using their zero order spectra with no need for advanced expensive software or device (as Matlab in MCR). The advantage of a-Factor method over the AFM is that the application of the last one is limited for the determination of binary mixture where one of the two component shows two maxima in the zero order absorption spectrum at two different wavelengths and the other component should not interfere at one of them; while the a-factor method has no limitation as it can utilize any spectrophotometric method to calculate the concentration of one component then calculate the others by a simple mathematical calculation. The MCR has the advantage of being more sensitive by enhancing the signal to noise ratio due to lack of derivatization. The TLC-spectrodensitometric method has the advantage of minimizing the cost of reagents and time for analysis. It also utilizes the merit of applying several sample bands on TLC plate, which may be more advantageous for regulatory quality control laboratories. In addition, the method is inexpensive and not requires certain types of stationary phases. As a final conclusion, the proposed methods proved to be accurate, precise and sensitive, and they have the advantage of being simpler as they do not require any sophisticated apparatus, as manufacturer HPLC method. The proposed methods could be easily applied in quality control laboratories as they show similar accuracy and precision compared to the manufacturer HPLC method for the simultaneous determination of sodium cromoglicate and fluorometholone, in addition to their lower cost.

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