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ORIGINAL RESEARCH PAPER

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Sensitive and validated TLC densitometry method coupled with fluorescence detection for quantitative determination of the newly co-formulated drugs, celecoxib and amlodipine besylate in tablet dosage form

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ABSTRACT

New, sensitive, rapid, cost-effective, and validated stability-indicating thin layer chromatographic (TLC) method coupled with fluorescence (FL) detection was developed for the quantitative analysis of celecoxib (CEL) and amlodipine besylate (AMLO) in their laboratory prepared binary mixture using the non-fluorescent TLC silica gel 60 plates. Ethyl acetate: diethylamine: 1-propanol (9:1:0.2, V/V) was used as a developing system. The retention factor (R_f) for each drug was 0.80 ± 0.03 and 0.44 ± 0.01 for CEL and AMLO, respectively. The plates were excited at 264 nm for the simultaneous FL measurement of CEL and AMLO, the calibration curves were linear over a concentration ranges of 30.0–300.0 ng/band and 15.0–150.0 ng/band with mean percentage recoveries of 99.80 ± 0.85 and 99.80 ± 0.77 For CEL and AMLO, respectively. The developed method was applied for the stability studies of the cited drugs in their laboratory prepared binary mixture and the forced degradation products were determined when present in presence of the pure drugs so the method can be considered as a stability-indicating one and it was validated as per ICH guidelines and proved to be accurate and precise.

KEYWORDS

celecoxib, amlodipine, TLC - densitometry, fluorescence detection mode, stability-indicating assay

1. INTRODUCTION

According to a recently constructed clinical study, it was concluded that patients suffering from osteoarthritis with a history of cardiovascular problems mostly hypertension have a comorbid relationship between osteoarthritis and hypertension [1]. It was found that approximately 40% of osteoarthritis patients also suffering from hypertension, as hypertension progresses, the blood vessels diameters become narrower and this leads to decreasing the blood supply to the joints and the surrounding protective cartilages thus worsen the prognosis over time [2]. Thus, a fixed-dose combination of celecoxib (CEL) and amlodipine besylate (AMLO) in tablet dosage form under the brand name (Consensi®) has been approved by FDA to manage both conditions, this novel combination leads to increasing the efficacy and patient adherence for the treatment protocol [3].

CEL, which is designated chemically as 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene-1-sulfonamide as illustrated in Fig. 1A [4] is a selective non-steroidal anti-inflammatory drug (NSAID). It inhibits the COX2 enzyme so it is a safe alternative for the non-selective (NSAIDs) in the management of pain and inflammatory disorders and osteoarthritis. As it maintains the normal prostanoid level in the stomach so it protects the stomach

Fig. 1. Chemical structure of (A) CEL and (B) AMLO

from the risk of ulcers and it has a lesser effect on hypertension in comparison with the other regular (NSAIDs) [5].

AMLO which is designated chemically as 3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate; benzene-sulfonic acid as shown in Fig. 1B [4] is a dihydropyridine derivative. And pharmacologically is a long-acting calcium-channel blocker used broadly for the treatment of cardiovascular conditions such as hypertension and angina either as a single agent or co-administered with other antihypertensive agents. It has also been co-administered with different drugs, for example, statins, diuretics, and anti-diabetics [6, 7].

Numerous analytical techniques have been established for the determination of CEL and AMLO alone or combined with other drugs as spectrophotometric [8–11], spectrofluorometric [12–15], high-performance thin-layer chromatography (HPTLC) [16–30], HPLC [31–33], and electrochemical methods [34, 35]. A few recently analytical techniques have been established for the simultaneous estimation of CEL and AMLO such as spectrophotometric [36], spectrofluorimetric [37], and HPLC [38, 39]. To date, no thin layer chromatographic (TLC) technique coupled with fluorescence (FL) detection mode has been reported for the simultaneous determination of CEL and AMLO, so our work aimed to the development of a simple, rapid, sensitive, economic, and validated TLC method coupled with FL detection mode for the separation of the two cited drugs in presence of their forced degradation products when present; moreover, we attempt to prove that our proposed method acts as a stability-indicating one.

2. EXPERIMENTAL

2.1. Materials and methods

Celecoxib (CEL) of purity 99.60% was obtained from (AMOUN, Cairo, Egypt), amlodipine besylate (AMLO) of

purity 99.51% was obtained from (Nile pharma, Cairo, Egypt) as gratis samples.

Celebrex capsules (Batch No 19271) labeled to contain 200 mg CEL (Pfizer, Cairo, Egypt) with an expiration date 12/2021 and Windipine tablets (Batch No 96002) labeled to contain 10 mg AMLO (Sanofi Aventis, Cairo, Egypt) with an expiration date 10/2022. Both preparations were purchased from the local market.

All solvents used except methanol were of analytical grade; methanol HPLC grade, 1-propanol and sodium hydroxide (Sigma-Aldrich, Germany), ethyl acetate (Riedel de Haen company, Germany), diethylamine (Loba Chemie company), hydrochloric acid and hydrogen peroxide solutions (Piochem company, Cairo, Egypt).

2.2. Instrumentation and chromatographic conditions

TLC coupled with FL detection mode has been performed over TLC silica gel 60 glass plates 20 × 10 cm with 0.25 mm (250 μm) thickness (E. Merck, Germany). The Samples were spotted in the form of distinct bands (3 mm in width) using CAMAG Linomat 5 auto-sampler with CAMAG microliter syringe (10 μL); (CAMAG, Muttentz, Switzerland). TLC scanner 3 densitometer, model 3 S/N 130319; (CAMAG, Muttentz, Switzerland). The performance of this equipment had been verified according to USP. Instrumental parameters were optimized for smooth working (scanning speed 20 mm/s; slit dimension 3.00 × 0.45 mm). Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (CAMAG, Switzerland), previously saturated with the optimized mobile phase that consists of ethyl acetate: diethylamine: 1-propanol (9:1:0.2, V/V) for 30 minutes at room temperature (RT). The plates were developed to a distance of 80 mm with a development time of 10 minutes. The plates were dried at RT. For the FL detection of the scanned TLC plates, the intensity of the emitted light was measured after excitation at 264 nm by mercury (Hg) lamp using optical filter K 320.

2.3. Standard solutions of CEL and AMLO

Stock solutions with a concentration of 2.0 mg/mL for each of AMLO and CEL were prepared by weighting 20.0 mg of each drug then each of them was transferred separately into 10 mL volumetric flasks, dissolved, and completed to the mark with methanol, 0.5 mL from each of prepared stock solutions was transferred separately into 10 mL volumetric flasks and completed to the mark with the same solvent to give a final concentration of 100.0 µg/mL for each of the studied drugs.

2.4. Construction of the calibration graph and analysis of pure bulk powders

Different aliquots from the above-prepared stock solutions under section 2.3 were transferred to a series of 10 mL volumetric flasks and completed to the mark with the same solvent then 1.0 µL from each solution was spotted in triplicate on the plates using CAMAG Linomat auto-sampler with CAMAG microliter syringe (10 µL) to give a concentration ranges of 30.0–300.0 ng/band and 15.0–150.0 ng/band for CEL and AMLO, respectively. The plates were developed and scanned under the previously stated chromatographic conditions under section 2.2. The calibration graphs for the drugs under study were constructed by plotting the average peak areas versus the corresponding concentrations in ng/band and the corresponding regression equations were derived.

2.5. Analytical application

2.5.1. Determination of CEL and AMLO in their laboratory prepared mixture. CEL and AMLO (200/10 mg) combined tablet dosage form was not accessible in the local Egyptian market; so we prepared a binary mixture at the same ratio by weighing individually the contents of ten Celebrex 200 mg capsules after evacuating the capsules then the contents of the ten capsules were mixed well and the amount of the powder equivalent to 200 mg of CEL was weighted. The same procedures were constructed for weighting the amount of powder equivalent to 10 mg of AMLO using Windipine 10 mg tablets after grinding well the ten tablets in a clean and dry mortar and pestle, then the two portions were mixed well and transferred to 25 mL volumetric flask that contains 15.0 mL methanol and dissolved well by using the ultrasonic bath for 15 minutes at 25 °C then the volume was completed to the mark with the same solvent and filtered using (0.45 µm) disposable syringe filter. 1.25 mL of the above-prepared solution was transferred to a 10 mL volumetric flask and completed to the mark with the same solvent then 0.3 µL was spotted in triplicate on the plates and developed using the mobile phase described under the chromatographic conditions under section 2.2. The nominal contents of the binary mixture were calculated from the corresponding regression equations.

2.5.2. Forced degradation studies. 1.25 mL aliquot which contains 10.0 mg of CEL and 0.5 mg of AMLO was

withdrawn from the solution prepared under section 2.5.1 that contains 8.0 mg/mL of CEL and 0.4 mg/mL of AMLO was transferred into a series of 10 mL stoppered volumetric flasks and used for each of the following forced degradation studies:

2.5.2.1. Hydrolytic degradation. To the above drug solution, 1.0 mL of 2 N NaOH, 1.0 mL of 2 N HCL, or 1.0 mL of water was added, then the flasks were left for different times at different temperatures; 1 hour at RT, 2 hours at 67 °C, and 12 hours at 67 °C, respectively. The three flasks were shielded from the light by wrapping in aluminum sheets. After the required time for each stress condition was spent, the solution that was subjected to alkali degradation was neutralized using HCL solution with the same strength as the NaOH used and in the case of the solution that was subjected to acid degradation, it was neutralized using NaOH solution with the same strength as HCL used after cooling. The solutions in the three flasks were completed to the volume using the same solvent, then 0.3 µL aliquots from each flask were spotted in triplicate on the plates and the plates were scanned as mentioned under section 2.2. The nominal contents of the aliquots were calculated from the corresponding regression equations.

2.5.2.2. Oxidative degradation. To the fourth flask 1.0 mL of 1.5 % H₂O₂ was added and left for 15 minutes at RT then the mixture was boiled on the water bath to expel excess H₂O₂. The resulting solution was quantitatively transferred to a 10 mL volumetric flask and completed to the mark with the same solvent and the procedures under section 2.5.2.1 were followed to determine the nominal contents of the aliquots.

2.5.2.3. Photo-degradation. The fifth flask was exposed to direct sunlight for 3 days then it was completed to the mark with the same solvent and it was proceeded as under section 2.5.2.1 to determine the nominal contents of the aliquots.

3. RESULTS AND DISCUSSION

Densitometry nowadays provided a smart solution for almost all chemistry problems. It has many applications in the field of pharmaceutical analysis and quality control laboratories even though it became an alternative tool for HPLC technique. As it gives the facility for shorter analysis time and decreases the consumption of the solvents compared with the HPLC technique. The development of HPTLC, the use of auto-samplers, sample application as a band instead of a spot and developing an incorporated chamber enhanced the sensitivity of the technique significantly to achieve quantitation limits in nanograms or picograms levels (depending on the detection mode whether in ultraviolet (UV) absorbance mode, fluorescence mode or mass mode) [40]. Our research aim is the development of a highly sensitive and selective TLC method for the simultaneous determination of the cited drugs in presence of their forced degradation products when present using the FL

detection mode. The use of FL detection mode greatly enhanced the sensitivity which exceeded the sensitivity that was achieved by the recently reported HPLC [38]. Regarding the assay of CEL, our developed method achieved higher sensitivity and in the case of AMLO, our method achieved slightly lower sensitivity to that was achieved by the reported HPLC method but with a wider range of linearity as shown in Table 1. Moreover, our method achieved higher sensitivity in comparison with that was achieved using the HPTLC methods in the literature for each of the two drugs under study as shown in Table 1.

3.1. Method optimization

To achieve good separation with symmetric sharp peaks and an acceptable R_f , the following parameters should be optimized;

3.1.1. Mobile phase system. Several trials for developing the mobile phase system were done taking into consideration the importance of developing an eco-friendly developing system as possible as we can. So we excluded the hazardous solvents from our choices such as toluene, chloroform, and dichloromethane which can cause a great hazard for the environment and human beings as toluene and dichloromethane are highly inflammable and chloroform is a carcinogenic agent for human. These solvents were used for constructing the developing system for separation of AMLO from other drugs in the previously reported TLC methods in literature as shown in Table 1. Many solvent systems containing different mixtures and ratios of ethyl acetate, methanol, and ammonia or ethyl acetate, and diethylamine were examined. The ternary solvent mixture that composed of ethyl acetate: diethylamine: 1-propanol (9:1:0.2, V/V) gave an optimum resolution with compact zones corresponding to the two cited drugs and gave sufficient separation between the cited drugs and the forced degradation products when present. The R_f values were 0.80 ± 0.03 and 0.44 ± 0.01 for CEL and AMLO, respectively as shown in Fig. 2.

3.1.2. Stationary phase. The non-fluorescent plate (TLC silica gel 60) was used for the FL detection mode which was used for the first time for the simultaneous determination of the two drugs under study. The idea originated from the problem which appeared upon using the fluorescent TLC silica gel 60 F_{254} plate for the FL detection mode of the cited drugs which is the appearance of a negative peak corresponding to (CEL) upon using 264 nm as an excitation wavelength and K320 as a cut off filter. But this problem did not appear with the detection of (AMLO) upon using 365 nm as an excitation wavelength and K400 as a cut off filter. Our assumption for the cause of this problem is that the fluorescent indicator (F_{254}) in the fluorescent plate which gives fluorescence upon excitation at short-wavelength ultraviolet light (254 nm) was the main reason for the inversion of the peak corresponding to CEL. So our method highlighted and solved this problem by using the non-fluorescent TLC plate and this can be applied to any

fluorescent compound that can be excited at a wavelength near that of the fluorescent indicator.

3.1.3. Optimum wavelength selection. Several excitation wavelengths were tested to achieve maximum FL intensity for both drugs. 264 nm was the optimum excitation wavelength that gave maximum FL intensity of the two drugs under study and guaranteed good sensitivity for the quantitative analysis of the two cited drugs.

3.1.4. Slit dimensions of scanning light beam. The slit dimensions of the scanning light beam should cover the band dimensions on the scanned track completely without the occurrence of any interference from adjacent ones. Different slit dimensions were tested and it was found that (3.00×0.45 mm) was the optimum slit dimensions which provided the best selectivity and the highest sensitivity.

3.1.5. Saturation time. For obtaining more reproducible results with good R_f values, the same degree of vapor saturation is recommended [40]. So, numerous saturation times ranging from 10 to 40 minutes were tried to select the most suitable time for saturation. It was commemorated that good results were attained beginning from 25 minutes; as a result, 30 minutes was used as an optimum saturation time.

3.1.6. Migration distance and development time. Since solvent gradient up the plate could be resulted from solvent de-mixing and as a consequence, separation and R_f values could be affected [40]. Therefore, we should standardize the mobile phase migration distance. A migration distance of 80 mm from the start was selected, as it achieved the optimum resolution; this distance of migration was achieved within 10 minutes.

3.2. Method validation

For validation of the proposed method, the following parameters; linearity and Range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, and selectivity were studied according to the ICH Guidelines Q2 (R1) [41]. The results are listed in Table 2.

3.2.1. Linearity and range. As shown in Figs 2A and 3, the concentrations of CEL and AMLO were proportional to the area under the peak of the separated bands, in concentration ranges of (30.0–300.0) and (15.0–150.0) ng/band for CEL and AMLO, respectively. When the same chromatographic conditions were performed using the fluorescent TLC plate (TLC silica gel 60 F_{254}) followed by Abs detection mode, the linear concentration ranges were (150.0–1400.0) and (50.0–600.0) ng/band for CEL and AMLO, respectively, as illustrated in Fig. 2B. These results revealed that using the FL detection mode enhanced the sensitivity significantly which reached 5 fold in the case of CEL and 3.3 fold regarding AMLO more than that obtained using Abs detection mode. Statistical analysis of the data revealed that the correlation coefficient (r) value was high and the values corresponding to the standard deviation and the percentage relative

Table 1. Summary of the reported chromatographic methods for the determination of CEL and AMLO and our proposed method

Analytes	Matrix	Mobile phase	Stationary phase	Quantitative range of our studied drugs	References
CEL	Dosage form.	n-hexane: ethyl acetate (6:4 v/v)	Precoated silica gel aluminum plate 60	200–2,000 ng/band	[22]
AMLO and TELM		Tetrahydrofuran: dichloromethane: methanol: ammonia solution (3.0:1.0:0.5:0.2 v/v)	F ₂₅₄ .	400–1,400 ng/band	[17]
AMLO, VAL, and HCTZ		Ethyl acetate:toluene: methanol: ammonia (50.5:23.5:23.5:2.5v/v)		0.5–9.0 µg/band	[23]
AMLO and AZIL		Chloroform:toluene: methanol: glacial acetic acid (7:1.5:1.5:0.5 v/v)		0.2–4.0 µg/band	[24]
AMLO, LOS, and HCTZ		Chloroform: methanol: acetone: formic acid (7.5 : 1.3 : 0.5 : 0.03 v/v)		500–3,000 ng/band	[16]
AMLO, LOS, and HCTZ		Chloroform:ethyl acetate: methanol: ammonia (4:4:2:0.2 v/v/v/v)		100–800 ng/band	[26]
AMLO and TELM		Ethyl acetate: methanol: ammonia: glacial acetic acid (7.5:1.5:1.0:0.2 v/v)		500–6,000 ng/band	[25]
AMLO and VAL		Toluene: methanol:acetic acid (7:3:0.1 v/v)		100–600 ng/band	[27]
AMLO and IREB		Chloroform: toluene: methanol: acetic acid (6:2.5:1.5:0.5v/v)		400–900 ng/band	[28]
AMLO and ENAL		Toluene: isopropanol: glacial acetic acid (GAA): methanol (6:2:0.6:0.5 v/v)		200–1,400 ng/band	[29]
AMLO and NEBIV		Methylene chloride: methanol: ammonia (8.5:1:0.5 v/v)		100–500 ng/band	[30]
AMLO, HCTZ, and ALISK	Dosage form	Ethyl acetate: methanol: ammonia (7.5:2.8:0.22 v/v)	Precoated silica gel aluminum plate 60	50–175 ng/band	[18]
AMLO, VAL, and HCTZ		Ethyl acetate: methanol: toluene: ammonia (7.5:3:2:0.8 v/v)	F ₂₅₄ .	100–600 ng/band	[19]
AMLO, VAL, and HCTZ		Chloroform: glacial acetic acid: n-butyl acetate (8:4:2 v/v)		0.2–0.6 µg/band	[20]
AMLO and RAMIP		Toluene: ethanol: acetone: ammonia in the ratio of (7:2:1:0.3 v/v)		1.4–7.6 µg/band	[21]
CEL and AMLO		Sodium phosphate buffer (pH 5.6): acetonitrile: methanol in a ratio 30:55:15 (v/v) as a mobile phase	Zorbax C18 (150 mm) reverse-phase (RP)-HPLC column	CEL: 50–500 µg/mL AMLO: 5–30 µg/mL	[38]
CEL and AMLO		Acetonitrile: potassium phosphate buffer (50 mM; pH 5.5), 60:40 (v/v) as a mobile phase	C18 reversed phase column (Thermo ODS Hypersil, 4.6 × 250 mm, particle size 5 µm)	CEL: 0.05–10 µg/mL AMLO: 0.05–2 µg/mL	[39]
CEL and AMLO		Ethyl acetate: diethylamine: 1-propanol (9:1:0.2, v/v)	TLC silica gel 60 glass plates for FL detection mode	CEL: 30.0–300.0 ng/band (µg/mL) AMLO:15.0–150.0 ng/band (µg/mL)	Our proposed method

CEL: celecoxib, AMLO: Amlodipine, TELM: telmisartan, VAL: valsartan, HCTZ: Hydrochlorothiazide, AZIL: azilsartan, LOS: losartan, IREB: irbesartan, ENAL: enalapril, NEBIV: nebivolol, ALISK: Aliskiren, RAMIP: ramipril.

Fig. 2. 3d densitograms of CEL and AMLO using (A) FL detection mode and (B) Abs detection mode

standard deviation were small. These data gave evidence of the good linearity of the calibration curves as shown in Table 2.

3.2.2. Limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ for the drugs under study were calculated as per ICH guidelines. The LOD and LOQ were separately estimated based on the calibration graphs. The standard deviation of the y -intercepts (σ) and slope of the regression lines (S) were utilized. Results of LOD and LOQ are illustrated in Table 2. LOD and LOQ had been calculated

from the calibration curves according to the following equations: $LOD = 3.3\sigma/S$ $LOQ = 10 \sigma/S$.

3.2.3. Accuracy. Accuracy was found to be 99.77 ± 0.39 and 100.44 ± 0.39 for standard CEL and AMLO, respectively as illustrated in Table 3. And 98.36 ± 0.81 and 99.82 ± 1.67 for CEL and AMLO, respectively in their laboratory prepared binary mixture as shown in Table 4. For further proving the accuracy of the proposed method, the reported HPLC method [38] was applied for the analysis of the drugs under study in their laboratory prepared binary mixture.

Table 2. Analytical parameters for simultaneous analysis of CEL and AMLO by the proposed TLC method using Abs and FL detection modes

Detection mode	TLC- Abs		TLC -FL	
Drugs	CEL	AMLO	CEL	AMLO
Parameters	Value		Value	
Concentration range (ng/band)	150.0–1400.0	50.0–600.0	30.0–300.0	15.0–150.0
Determination coefficient (r ²)	0.9999	0.9999	0.9999	0.9999
S.D	0.90	0.60	0.85	0.77
% RSD	0.90	0.60	0.85	0.77
Slope	5.5045	4.8137	25.688	42.342
Intercept	1397.4	150.58	1841.3	474.67
Standard deviation of intercept	64.64	21.78	74.78	51.63
Limit of detection (LOD), ng/band	41.2	14.9	9.6	4.0
Limit of quantitation (LOQ), ng/band	124.7	45.2	29.1	12.2

Fig. 3. Calibration curves of (A) CEL and (B) AMLO using FL detection mode

Table 3. Simultaneous determination of CEL and AMLO in pure form by the proposed TLC method coupled with FL detection mode

CEL		AMLO	
Conc (ng/band)	% Found ^a	Conc (ng/band)	% Found ^a
250.0	100.22	35.0	100.81
130.0	99.59	65.0	100.49
70.0	99.50	125.0	100.02
Mean ± SD	99.77 ± 0.39	Mean ± SD	100.44 ± 0.39

^a Average of three determinations.

The results were compared statistically with that obtained from our proposed method using the following parameters; mean recoveries, student's *t*-test, and variance ratio *F*-test.

Table 4. Determination of CEL and AMLO in their laboratory prepared binary mixture that contains 200 mg CEL and 10 mg AMLO using the proposed TLC method coupled with FL detection mode

Drug	CEL	AMLO
Conc (ng/band)	300.0	15.0
% Recovery ^a ± SD	98.36 ± 0.81	99.82 ± 1.67

^a Average of three determinations.

Table 5. Statistical comparison of the results obtained from the analysis of the laboratory prepared mixture using our proposed method and the reported HPLC method [38]

Detection mode	TLC-FL		The reported HPLC method [38]	
	CEL	AMLO	CEL	AMLO
Drug				
Mean % recovery ^a	98.36	99.82	97.67	97.33
SD	0.81	1.67	0.33	0.39
<i>N</i>	3	3	3	3
<i>t</i> (2.77) ^b	1.39	2.50		
<i>F</i> (19) ^b	5.83	18.45		

^a Average of three determinations.^b Tabulated *t* and *F* values at *P* = 0.05.

Table 6. Assessment of the intra-day and inter-day precision of the proposed TLC method coupled with the FL detection mode for simultaneous determination of CEL and AMLO in pure form

CEL			
Drug	Conc (ng/band)		
	30.0	100.0	200.0
Intraday precision	99.59 ± 1.25	99.75 ± 0.59	100.81 ± 0.58
Inter day precision	99.91 ± 1.34	100.66 ± 1.61	100.97 ± 0.70
AMLO			
Drug	Conc (ng/band)		
	15.0	50.0	100.0
Intraday precision	99.83 ± 1.44	100.08 ± 0.61	101.15 ± 0.54
Inter day precision	100.59 ± 1.68	99.54 ± 1.20	100.98 ± 0.85

The statistical comparison between the results that obtained from the developed and the reported methods revealed that there was no significant difference between the performance of our developed method and the reported method as illustrated in Table 5.

3.2.4. Precision. Precision was evaluated through replicate determination of CEL and AMLO standard solutions at three concentration levels on consecutive three intervals on the same day for intra-day precision and over a period of

three consecutive days for inter-day precision. The low standard deviations of the different measurements revealed that the proposed method has good precision as illustrated in Table 6.

3.2.5. Specificity and selectivity. For the determination of the specificity of the proposed method, the purity of the peaks of the drugs under study was evaluated. This was done by using the TLC scanner that recorded the absorption spectrum at several points across each peak as illustrated in

Fig. 4. Overlaid spectra of (A) CEL and (B) AMLO

Fig. 4. The high value of correlation coefficient that reached 0.9996 between the spectra recorded at peak start and peak maximum ($r_{s,m}$) and that between the spectra recorded at peak-end and peak maximum ($r_{e,m}$) was 0.9997. The mathematic interpretation of the software revealed that the peak is pure. The selectivity of our proposed method was further proved by its ability to determine the forced degradation products when present in presence of the pure drugs.

3.2.6. Robustness. Method robustness was evaluated by minor yet deliberate variations within the optimal

parameters of the analytical method during its application on 30.0 and 15.0 ng/band for standard CEL and AMLO, respectively. Variation in composition of the mobile phase (± 0.1 mL) for each component, period for chamber saturation (± 5 min), and migration distance (± 5 mm) were cautiously investigated. The effects of these variations on R_f values as well as the area under the peak were assessed by calculating the standard deviation (SD) for the parameter under study in comparison with the optimal conditions. It was observed that the calculated standard deviations of the different measurements were all < 2 ; this proved that our

Fig. 5. (A, B) Chromatograms of CEL and AMLO under different stress conditions using FL detection mode; (A) base (2 N NaOH at RT for 1 hour) hydrolysis, (B) acid (2 N HCL at 67 °C for 2 hours) hydrolysis, (C) neutral (at 67 °C for 12 hours) hydrolysis, (D) oxidative (1.50% v/v H_2O_2 at RT for 15 minutes), and (E) photo (direct sunlight for 3 days) degradation

proposed method has good Robustness and repeatability for analysis of the cited drugs.

3.3. Analysis of the pharmaceutical sample

The area under the peak from the CEL and AMLO standard solutions and laboratory prepared binary mixture solutions were utilized for quantifying the amounts of the cited drugs in their laboratory prepared binary mixture in ratio 20:1 for CEL and AMLO, respectively. Our proposed method results were compared statistically to that of the reported method [38]. There was no significant difference between the two methods was found at 95% confidence level as illustrated in Table 5, this showed that our proposed technique could be reliably utilized for the simultaneous determination of CEL and AMLO in its pharmaceutical dosage form without the occurrence of any interference from excipients.

3.4. Results of forced degradation studies

Hydrolytic, oxidative, and photo forced degradation were investigated. In all cases, CEL was stable and completely recovered and no degradation products were observed concerning this drug under the studied conditions as shown in Fig. 5A. Also, AMLO was stable under the photo, alkali, and neutral hydrolysis conditions while appreciable degradation was detected in the case of oxidative and acidic hydrolysis. AMLO is liable to acidic hydrolysis as 52% of the

drug was recovered with no peaks corresponding to degradation products were detected while in case of oxidative degradation 20% of the drug was recovered also without observation of any peaks corresponding to degradation products as shown in Fig. 5B. So it could be concluded that; the forced degradation products of AMLO are not fluorescent which is in agreement with the previously reported study by [13]. To exclude any doubt for any possible interference from any degradation product, the peak purity test for the parent drugs peaks was performed as previously mentioned under section 3.2.5. For further contribution to detecting the degradation products in acidic and oxidative degradation, the Abs detection mode was used using TLC silica gel 60 F₂₅₄ plates and the same chromatographic condition except using deuterium lamp, and the plates were scanned at 239 nm taking into consideration that larger injection volume (1.0 μ L) was applied from the same solutions used for stability study using the FL detection mode due to lower sensitivity of Abs detection mode. Regarding AMLO acidic hydrolysis, one peak corresponding to one degradant was detected while in the case of oxidative degradation, two peaks corresponding to two degradation products were detected as shown in Fig. 6. These results added further evidence on the selectivity of the developed method for the drugs under study, so it could be considered as a stability-indicating one. The results were summarized in Table 7.

Fig. 6. Chromatograms of CEL and AMLO under different stress conditions using Abs detection mode; (A) base (2 N NaOH at RT for 1 hour) hydrolysis, (B) acid (2 N HCL at 67 °C for 2 hours) hydrolysis, (C) neutral (at 67 °C for 12 hours) hydrolysis, (D) oxidative (1.50% v/v H₂O₂ at RT for 15 minutes), and (E) photo (direct sunlight for 3 days) degradation

Table 7. Summary of the results of stability study of CEL and AMLO using various stress conditions

Degradation type	Condition	% recovered		R _f of degradation products	
		CEL	AMLO	CEL	AMLO
Basic	2 N NaOH at RT for 1 hour	Stable	Stable	Not detected	Not detected
Acidic	2 N HCL at 67 °C for 2 hours	Stable	52%	Not detected	0.63
Neutral	At 67 °C for 12 hours	Stable	Stable	Not detected	Not detected
Oxidative	1.50 % v/v H ₂ O ₂ at RT for 15 minutes	Stable	20%	Not detected	0.58, 0.68
Photo	Direct sunlight for 3 days	Stable	Stable	Not detected	Not detected

4. CONCLUSION

The newly developed TLC method coupled with FL detection mode could be considered as fast, direct, sensitive, and specific one for simultaneous estimation of CEL and AMLO in pharmaceutical formulation. Also the facility to apply sample directly without any pretreatment, critical reaction, or any prior sophisticated steps made this method valid for application in quality control laboratories. The developed method showed stability-indicating potential as it can satisfactorily quantify CEL and AMLO simultaneously with the forced degradation products when present.

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