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ORIGINAL RESEARCH PAPER

Application of the green analytical procedure index to the simultaneous analysis of co-formulated tinidazole and ciprofloxacin in pure form, tablet dosage form, and human plasma using an environmentally friendly micellar high-performance thin-layer chromatographic technology

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Abstract

In individuals with chronic refractory pouchitis and other gastrointestinal disorders, the combination of ciprofloxacin (CIP) and tinidazole (TIN) is beneficial and safe. As a result, a green micellar high-performance thin-layer chromatographic (HPTLC) approach for the immediate analysis of TIN and CIP in pure, spiked human plasma, and co-formulated tablet dosage form has been developed. It is rapid, extremely easy, sensitive, cost-effective, and environmentally friendly. The stationary phase was Merck aluminum HPTLC plates covered with silica gel 60 F_{254}, while the mobile phase was acetone–ethanol–2% watery sodium dodecyl sulfate (3:4:2, *V/V*). For quantification of both medications, the densitometric scanner was set at 310 nm. For TIN and CIP, this chromatographic separation yielded symmetric, compact peaks with R_F values of (0.22 ± 0.009) and (0.42 ± 0.007), respectively. At 310 nm, the separated spots were densitometrically scanned. For TIN and CIP, the detection thresholds were 6.7 ng/band and 25.03 ng/band, respectively. For TIN and CIP, the quantification limits were 20.3 ng/band and 75.25 ng/band, respectively. The approach was validated according to International Council for Harmonisation (ICH) principles and then used to determine the researched medicines in their various pharmaceutical dosage forms, and human plasma yielding an exceptional percent of recovery. In terms of precision and accuracy, the results were in great accordance with the published approach. This method is suitable for the sequential analysis of the two drugs in pure form, tablet dosage forms, and spiked human plasma due to its simplicity, speed, greenness, robustness, and low cost.

Keywords Tinidazole · Ciprofloxacin · High-performance thin-layer chromatography (HPTLC) · Tinifloxacin tablet · Human

Abbreviations

TIN	Tinidazole
CIP	Ciprofloxacin
HPTLC	High-performance thin-layer chromatography
GAPI	Green analytical procedure index

1 Introduction

Tinidazole (TIN, Fig. 1) is a chemical compound, 1-[2-(ethylsulfonyl)ethyl]-2-methyl-5-nitro-1H-imidazole, and has a specific antimicrobial activity against anaerobic bacteria. TIN, which was developed in 1972, is one of the most important nitroimidazole antibiotics. TIN is a prodrug that produces a free nitro radical when its nitro group is reduced in trichomonas via a ferredoxin-mediated transport pathway. The antiprotozoal activity of TIN is due to the free nitro radical it generates [1]. TIN is effective against a variety of microorganisms, including *Helicobacter pylori, Trichomonas vaginalis, Giardia,* and amoebic dysentery [2].

Ciprofloxacin (CIP, Fig. 1), chemically 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid, is a broad-spectrum synthetic fluoroquinolone

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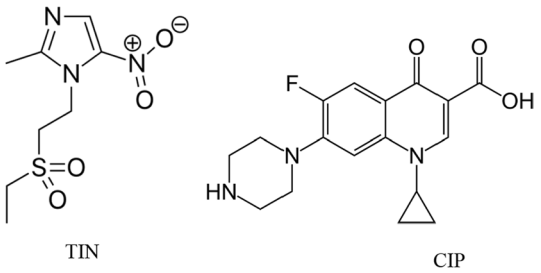

Fig. 1 The chemical structures of the analyzed compounds

antibiotic developed by Bayer in 1983 and approved for medicinal use in the United States by the Food and Drug Administration in 1987 [3]. CIP has a great antibiotic action against both Gram-positive and Gram-negative bacteria, like *Pseudomonas aeruginosa, Shigella* spp., *Escherichia coli,* and *Neisseria* [3]. Its antibiotic action is due to its ability to inhibit bacterial DNA gyrase and bacterial DNA topoisomerase, which are essential for bacterial replication [3].

The combination of TIN and CIP is quite useful in the treatment of a variety of bacterial and protozoal diseases. Chronic refractory paucities [4], surgical prophylaxis, surgical wound infections, gynecological infections, prophylaxis in gynecological procedures, respiratory tract infections, ear, nose and throat (ENT) infections, dermatological infections, and intra-abdominal infections were all treated with the mixture [5, 6].

This effective medication combination was separated and quantified using a variety of chromatographic methods, whether in pure form or pharmaceutical dosage forms. Spectrophotometric methods [7–9], high-performance liquid chromatography–ultraviolet (HPLC–UV) [10–14], and polarography [15] are among the proposed analytical approaches.

To our knowledge, no high-performance thin-layer chromatographic (HPTLC) approach for the instantaneous analysis of the examined drug combination has been established as of date.

HPTLC techniques, on the other hand, are a promising alternative to common HPLC techniques in some significant analyses, as HPTLC analysis can separate and estimate many analytes in a short time when compared to HPLC, and HPTLC methods use very small sample injection and mobile phase volumes when compared to traditional HPLC analysis, in addition to the possibility of recycling the mobile phase by using it more than once. Because traditional chromatographic procedures still employ huge amounts of non-degradable and ecologically harmful organic solvents, green environmental analysis approaches have become more important in several pharmaceutical research domains.

For these reasons, we developed a green micellar HPTLC method for the simultaneous assessment of the examined drug combination in pure forms and medicinal dosage formulations. HPTLC is an analytical technique that has been utilized to analyze a variety of pharmacological combinations [16–22] as well as stability-indicating processes [23–25].

2 Experimental

2.1 Instrumentation

CAMAG-Korea provided a HPTLC apparatus, which included a semi-automatic sample injection system for sampling under a nitrogen stream, a Hamilton® 100 µL sampling syringe (Bonaduz, Switzerland), and a CAMAG densitometer scanner. HPTLC plates coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany, 20 × 10 cm and 250 µm thickness) were used as the stationary phase. Acetone, ethanol, and 2% watery sodium dodecyl sulfate (3:4:2, *V/V*) were used in the development phase. Before sampling, all HPTLC plates were heated to 60 °C for 15 min. The chromatogram progressed to a distance of 9 cm in the TLC chamber (20 × 20 cm) at room temperature using the linear ascending mode; the chamber was thoroughly saturated by the developing phase for 30 min before the linear chromatogram progressed. The chromatogram took roughly 5 min to complete. Finally, the TLC plates were scanned using a CAMAG TLC Scanner 3 densitometric at 310 nm.

2.2 Reagents and materials

Organo Pharma kindly provided authentic TIN (99.9% purity) and CIP (99.8% purity) standards (El Obour industrial zone, Egypt). All the reagents used in the experiment were of analytical grade purity. Acetone, ethanol, ethyl acetate, sodium dodecyl sulfate, and ammonia (25%) were bought from the company El Gomhoria Chemical Co. (Cairo, Egypt).

2.3 Pharmaceutical formulation

Tinifloxacin tablet (batch No. T310421), qualified to contain 500 mg of CIP/tablet, and 600 mg TIN /tablet, was manufactured by Organo Pharma (Obour city, Egypt).

2.4 Preparation of stock standard solutions

Stock standard TIN solutions (100 µg mL^{-1}) were made by dissolving accurately weighted 10 mg of TIN powder in a 100-mL volumetric flask, diluting with roughly 25 mL ethanol, fully dissolved, and then adding ethanol to the mark.

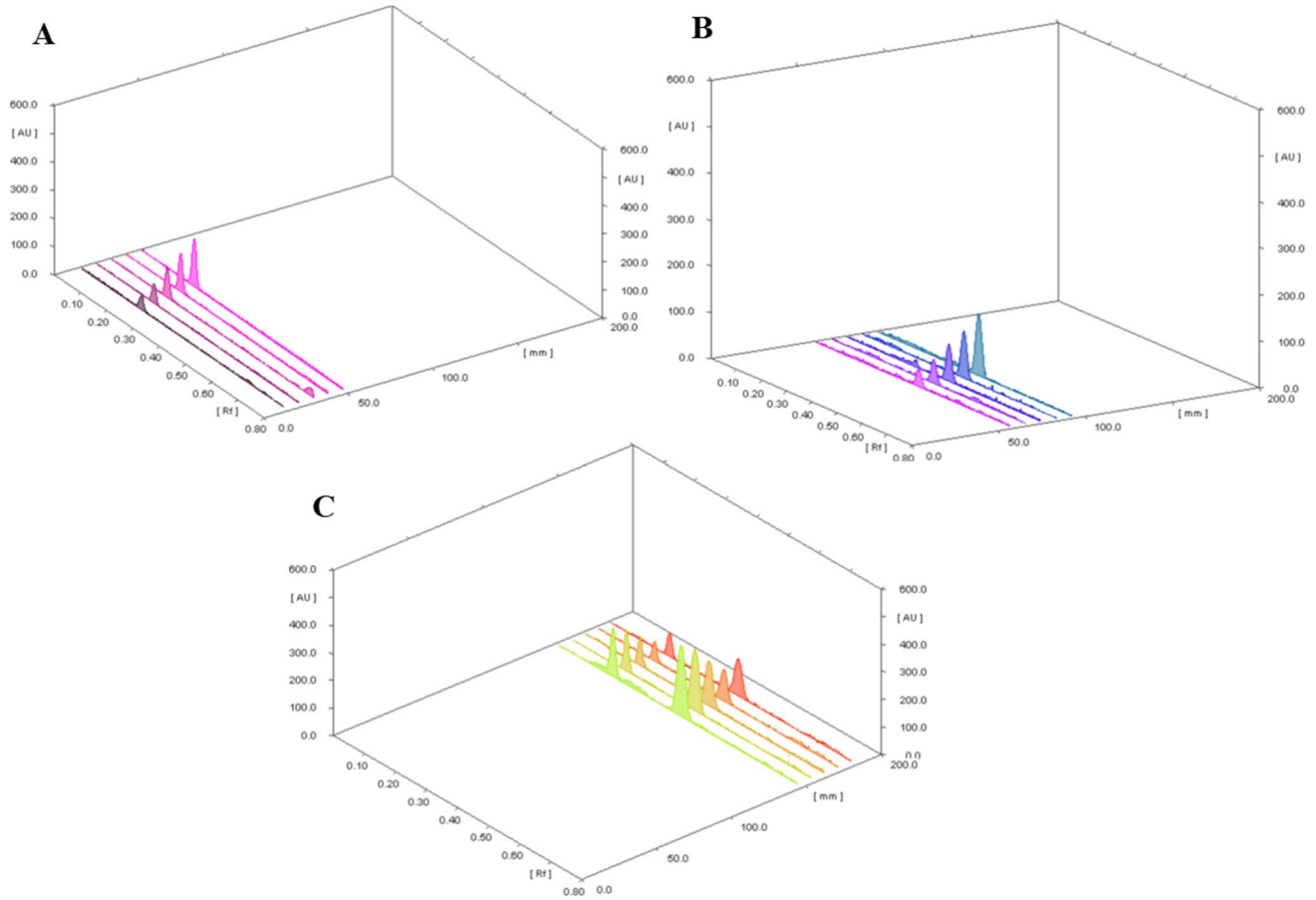

Fig. 2 A typical 3D HPTLC calibration chromatogram of **A** TIN (25, 100, 250, 500, and 1000 ng/spot), **B** CIP (80, 100, 250, 500, and 1000 ng/spot), and **C** mix of TIN and CIP, using acetone–ethanol–2% watery sodium dodecyl sulfate (3:4:2, *V/V*)

Furthermore, stock standard solutions of CIP (100 µg mL^{-1}) were made in the same manner, by properly weighing 10 mg of CIP powder into a 100-mL volumetric flask, diluting with roughly 25 mL ethanol, fully dissolved, and then adding ethanol to the mark.

2.5 Calibration graphs

Various aliquots of the standard stock solutions (1–10 µL of TIN and CIP) were simply spotted on TLC plates to achieve a final drug concentration of 25–1000 ng/band for TIN and 80–1000 ng/band for CIP, respectively. The calibration graphs were created by graphing the area under the peaks versus the drug amount.

2.6 Procedure for pharmaceutical formulation

Ten Tinifloxacin tablets were weighed accurately, finely powdered, and mixed thoroughly. An accurate quantity equivalent to the content of one tablet (600 mg TIN and 500 mg CIP) was weighted and transferred to a 100-mL volumetric flask, and extracted three times with 25 mL ethanol, The contents of the flask were swirled, sonicated for 5 min each time, and filtrated to a 100-mL volumetric flask; then the volume was completed to 100 mL with ethanol to obtain a final solution with concentrations (6 mg mL^{-1} TIN and 5 mg mL^{-1} CIP). The prepared solution was spotted on the TLC plates in different volumes to obtain the final substance amount within the calibration range. The general analytical procedure was applied to the tablet extraction.

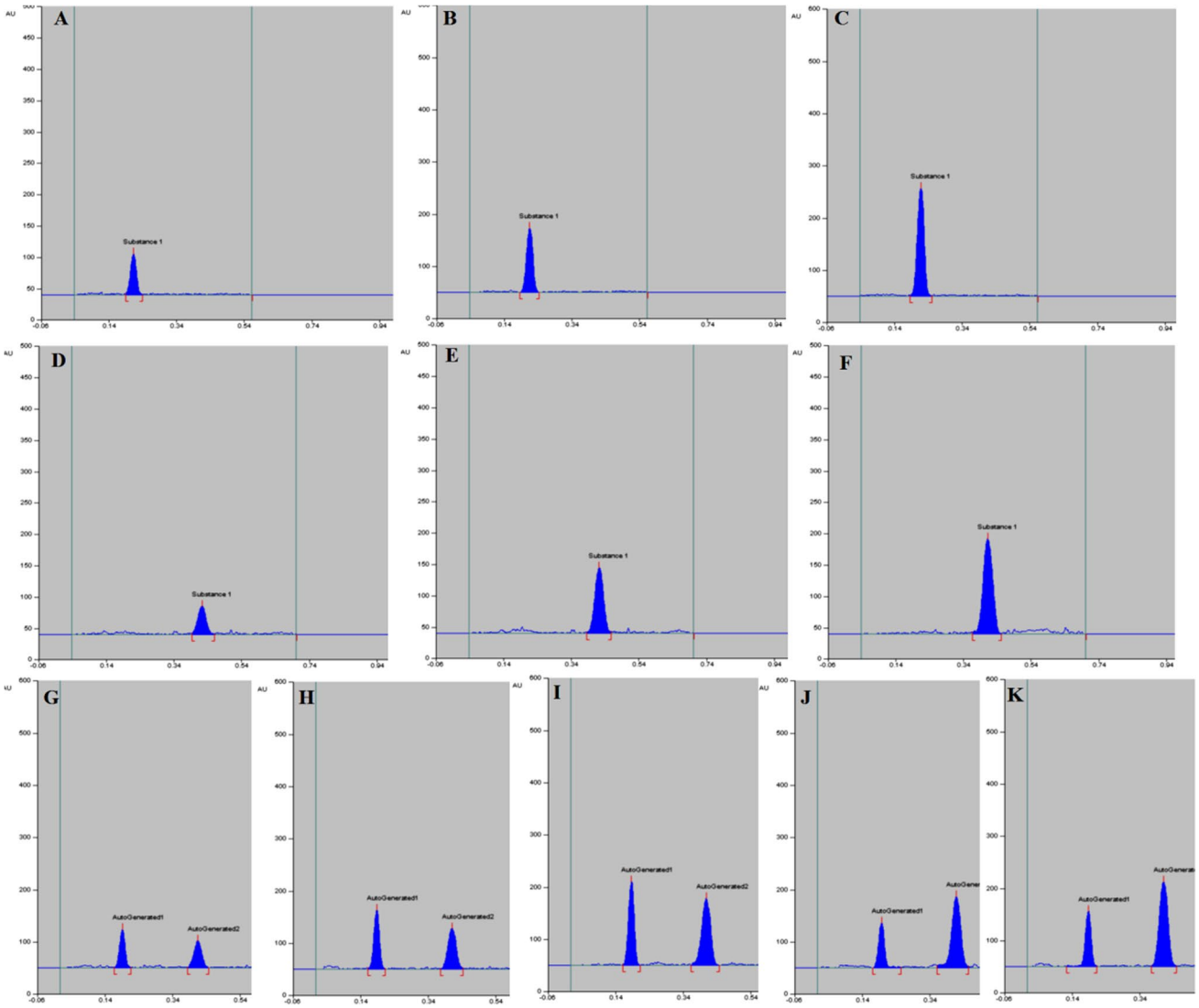

Fig. 3 A typical 2D HPTLC calibration chromatogram of **A** TIN (60 ng/band), **B** TIN (250 ng/band), **C** TIN (750 ng/band), **D** CIP (80 ng/band), **E** CIP (450 ng/band), **F** CIP (900 ng/band); laboratory-prepared mixtures of **G** TIN (85 ng/band), CIP (75 ng/band), **H** TIN (250 ng/band), CIP (250 ng/band), **I** TIN (600 ng/band), CIP (500 ng/band), **J** TIN (250 ng/band), CIP (750 ng/band), **K** TIN (400 ng/band), CIP (850 ng/band), using acetone–ethanol–2% watery sodium dodecyl sulfate (3:4:2, *V/V*)

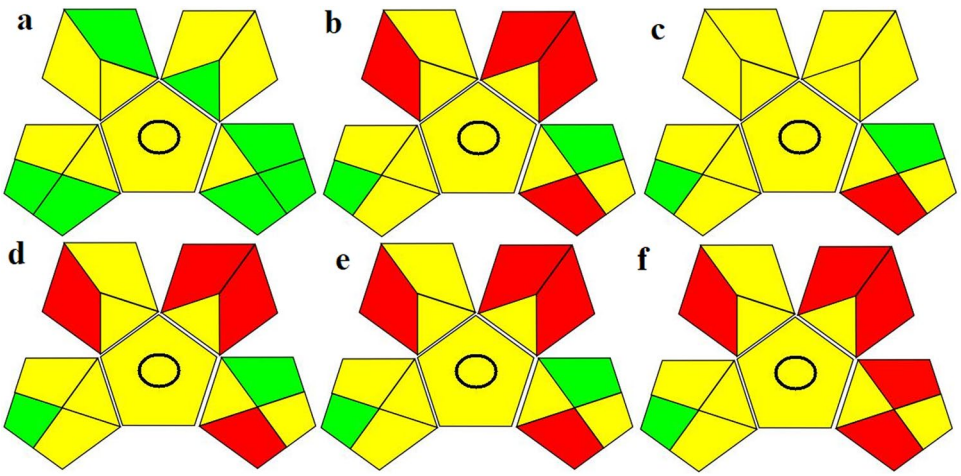

Fig. 4 GAPI pictograms for **a** the proposed method and previously reported methods, **b** for Ref. [10], **c** for Ref. [11], **d** for Ref. [12], **e** for Ref. [13], and **f** for Ref. [14]. The red zones represent high ecological impact, yellow zones represents lower impact, and green zones represent safe effect to environment

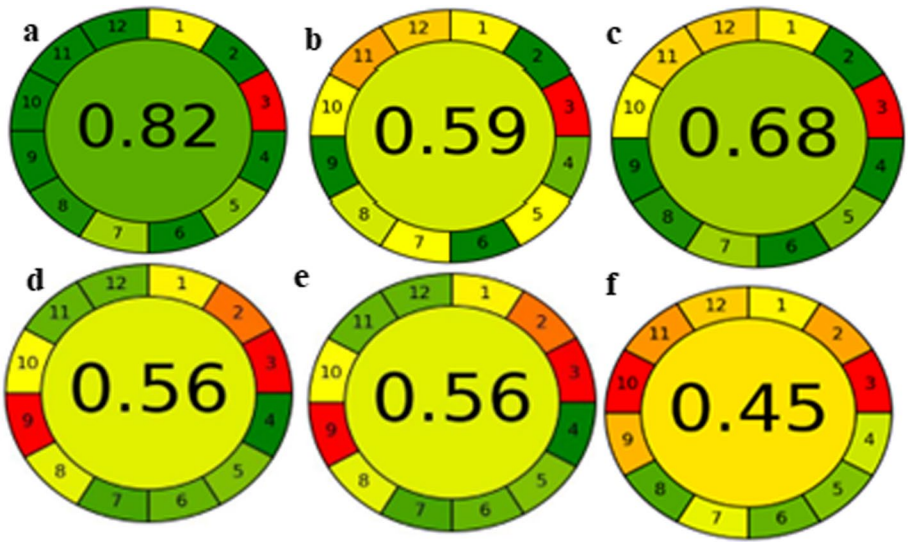

Fig. 5 AGREE pictograms for **a** the proposed method and previously reported methods, **b** for Ref. [10], **c** for Ref. [11], **d** for Ref. [12], **e** for Ref. [13], and **f** for Ref. [14]

2.7 Human plasma spiked procedure

According to institutional procedures, plasma samples were collected from normal, healthy, male human volunteers at Zagazig University Hospital (Zagazig, Egypt). An aliquot of 5.0 mL drug-free human plasma sample was transferred to a heparinized tube and vortex-mixed for 2 min at 2000 rpm before centrifugation at 4000 rpm for 30 min. 1.0 mL of drug-free plasma (supernatant) was spiked with 1 mL of stock standard solution into a 10-mL stoppered calibrated tube. Then begins the precipitation of plasma proteins. An aliquot of 2 mL of acetonitrile was added, then completed to 10 mL with distilled water. The tube contents were then centrifuged at 4000 rpm for roughly 15 min. To obtain different solutions within the concentration range of the tested medicines, varied quantities of the resultant supernatant were transferred to a series of 10-mL volumetric flasks and diluted to the mark with ethanol. Following that, the standard analytical method was performed. The drug-free blood sample was treated in the same way as the drug-treated blood sample but without the drug.

3 Results and discussion

3.1 HPTLC method

The therapeutic combination of TIN and CIP has a significant impact and is widely prescribed to treat a variety of diseases caused by bacteria or protozoa such as chronic refractory pouchitis [4]. The suggested HPTLC method proved to be successful in separating the two co-formulated medicines in pure form or in tablet pharmaceutical formulations. For separation and quantification of the two medicines examined, several reagent mixes were used as mobile phases. The separation of the examined pharmaceuticals was achieved using a mobile phase comprising of acetone, ethanol, ethyl acetate, and sodium dodecyl sulfate. Other mobile phases that have been studied have resulted in unsatisfactory separation of the analytes. As a result, we found that a mobile phase made up of acetone, ethanol, and 2% watery sodium dodecyl sulfate (3:4:2, *V/V*) yielded sharp, symmetric, and non-tailed analytes peaks. The separated medicines showed R_F values of (0.22 ± 0.009) and (0.42 ± 0.007) for TIN and CIP, respectively, after being eluted by the selected mobile phase for 5 min (Figs. 2 and 3). For investigation, the densitometric scanner was set to 310 nm. Furthermore, it was discovered that activating the HPTLC plates at 60 °C for 10 min before spotting the sample improved both peak form and repeatability of the suggested approach.

Furthermore, the selected mobile phase comprises a high proportion of sodium dodecyl sulfate, resulting in

Table 1 Analytical parameters for the analysis of TIN and CIP by the proposed HPTLC method

Parameter	TIN	CIP
Concentration range (ng/spot)	25–1000	80–1000
Correlation coefficient (*r*)	0.999	0.999
R_F	0.22	0.42
Determination coefficient (r^2)	0.999	0.999
Slope	6.46	4.23
Intercept	−19.68	−4.1
SD of the intercept (*S*a)	13.11	31.87
SD of slope (*S*b)	0.024	0.057
RSD of the slope (%)	0.37	1.35
Limit of detection (ng/band)	6.7	25.03
Limit of quantification (ng/band)	20.3	75.34

Table 2 Evaluation of the accuracy of the proposed HPTLC procedure for the determination of TIN and CIP at five concentration levels within the specified range

Sample number	Taken (ng/band)	TIN		CIP	
		Found (ng/band)	% Recovery[a] ± SD	Found (ng/band)	% Recovery[a] ± SD
1	150	150.00	100.00 ± 0.34	149.50	99.70 ± 0.13
2	250	249.00	99.60 ± 0.67	251.10	100.40 ± 0.25
3	400	398.00	99.50 ± 0.54	397.30	99.30 ± 0.35
4	500	502.50	100.50 ± 0.69	495.50	99.10 ± 0.65
5	800	799.00	99.88 ± 0.37	801.50	100.19 ± 0.99
Mean		99.90		99.71	
SD		0.39		0.56	
RSD		0.39		0.56	
RE		0.10		0.29	

[a]Mean of three replicate measurements

SD Standard deviation, *RSD* Relative standard deviation, *RE* Relative error

Table 3 Evaluation of the intra-day and inter-day precision of the proposed HPTLC method for the determination of TIN and CIP in pure form

Precision level	Conc. (ng\spot)	TIN		CIP	
		% Recovery[a] ± SD	RSD	% Recovery[a] ± SD	RSD
Intra-day	250	100.1 ± 0.30	0.30	99.8 ± 0.40	0.40
	500	99.9 ± 0.70	0.70	100.3 ± 0.29	0.29
	750	99.8 ± 0.22	0.22	100.4 ± 0.57	0.57
Inter-day	250	99.9 ± 0.40	0.40	100.6 ± 0.61	0.61
	500	100.6 ± 0.87	0.87	99.9 ± 1.04	1.04
	750	99.9 ± 1.29	1.29	100.1 ± 0.50	0.50

[a]Mean of three replicate measurements

SD standard deviation, *RSD* relative standard deviation

a micellar mobile phase, which improves the sensitivity of the proposed analytical approach by forming micellar structures with the analytes. Furthermore, because most analysts want to employ environmentally friendly methods in drug analysis, our approach differs from other typical HPTLC methods by using micellar green mobile phase.

3.2 Evaluation of greenness of the HPTLC analytical method

There is now a variety of tools available to assess and compare the greenness of various analytical techniques. In comparison to the prior analytical eco-scale [26], the new green analytical procedure index (GAPI) [26, 27] has the distinct benefit of spanning the whole analytical method. It has five pentagrams, each defining a stage in the analytical approach, such as sample collection and preparation, reagents, and solvents, applied instruments, and the analytical technique's purpose. GAPI has three color codes, with red indicating a high environmental hazard and yellow and green indicating a lesser hazard and improved greenness. The suggested HPTLC approach was compared to five existing processes that had previously been published. Figure 4 depicts the GAPI which compares the proposed methodology to five previously published analytical methods for the quantification of TIN and CIP. Three of these

Table 4 Determination of TIN and CIP in laboratory-prepared mixtures using the proposed HPTLC method

Mix No.	Conc. (ng/band)		% Recovery[a]	
	TIN	CIP	TIN	CIP
1	300	150	100.1	99.9
2	400	200	99.7	100.0
3	600	500	99.6	99.9
4	800	400	99.5	100.2
5	250	250	100.1	100.4
6	500	250	99.9	100.3
7	1000	500	99.5	99.9
8	1000	250	100.4	99.7
	Mean		99.85	100.04
	SD		0.33	0.24

[a]Mean of three replicate measurements

SD standard deviation

Table 5 Robustness study of the proposed HPTLC method for the determination of TIN and CIP (100, 250 ng/spot) in pure form

Variation Effect of the mobile phase composition	Conc. (ng/spot)	TIN % Recovery[a] ± SD	CIP % Recovery[a] ± SD
Acetone, ethanol, 2% watery sodium dodecyl sulfate (3:4:2, *V/V*)	100	99.70 ± 0.30	99.17 ± 0.95
	250	99.13 ± 0.55	99.87 ± 0.88
Acetone, ethanol, 2% watery sodium dodecyl sulfate (3.5:3.5:2, *V/V*)	100	98.90 ± 0.60	99.37 ± 0.45
	250	99.23 ± 0.47	99.67 ± 0.90
Acetone, ethanol, 2% watery sodium dodecyl sulfate (2.5:4.5:2, *V/V*)	100	99.00 ± 0.72	98.53 ± 0.83
	250	98.30 ± 0.98	99.03 ± 1.30

[a]Mean of three replicate measurements

SD standard deviation

procedures [12–14] employed acetonitrile as an organic solvent, whereas one approach [10] used methanol. As can be seen in Fig. 4a, the suggested technique contains seven green and eight yellow pentagrams, with no red pentagrams in its GAPI. The previously disclosed approaches (b, c, d, e, and f), on the other hand, contain 4, 1, 4, 4, and 5 red pentagrams, respectively, indicating substantial environmental risks. When compared to other previously published chromatographic techniques, the suggested HPTLC method is a green eco-friendly approach, according to the GAPI pentagrams. The main differences from previous procedures was the absence of the extraction step, which is the most inconvenient stage for most analysts, the use of green micellar solvents, the use of lower energy consumption instrumentation, and lower waste production, which can be easily recycled by using it for elution multiple times with multiple TLC plates.

AGREE [28] is another assessment tool that has been recently introduced on the color code based in GAPI. The main difference from GAPI is that it was based on the twelve green analytical chemistry (GAC) principles. AGREE shows a clock-shaped pictogram, the perimeter of which is divided into twelve sections, each corresponding to a GAC principle. The center of the pictogram shows a numerical value estimating the ecological impact, where the closer to 1, the better is the impact. As shown in Fig. 5, AGREE shows low ecological impact as expressed by the numerical 0.82 value. The perimeter is almost greener, except for the third GAC principle concerned with off-line sampling which is unavoidable as clarified in GAPI pictogram discussion. In the case of using the proposed method for pharmaceutical dosage form analysis, the method would be totally green due to the absence of any required organic solvents. The use of low energy HPTLC equipment, its higher throughput, and simple sample preparation procedures without the need for derivatizing agents account for the better environmentally friendly behavior of the proposed methodology.

3.3 Validation of the proposed HPTLC method

The HPTLC technique was fully verified for accuracy, precision, linearity, limit of quantification (LOQ), limit of detection (LOD), robustness, and selectivity in accordance with the International Council for Harmonisation (ICH) [29].

3.3.1 Linearity and range

The linearity of the proposed HPTLC technique was tested using six concentration points (25, 100, 250, 500, 700, and 1000 ng/spot for TIN and 80, 100, 250, 500, 750, and 1000 ng/spot for CIP), with each concentration being repeated three times and the mean of the three values being determined. The calibration graphs for the substances under investigation were created by graphing the area under the peak vs the relevant drug concentration.

The statistical treatment of the data using regression analysis established the analytical parameters (Table 1).

$$A_{TIN} = 6.46C - 19.68 \qquad r = 0.999 \qquad \text{(linear regression of TIN)}$$

$$A_{CIP} = 4.23C - 4.1 \qquad r = 0.999 \qquad \text{(linear regression of CIP)}$$

where A represents the peak area, C represents the drug concentration in ng/band, and r represents the correlation coefficients.

The two formulas, $\mathrm{LOD} = 3.3\sigma/S$ and $\mathrm{LOQ} = 10\sigma/S$ according to ICH guidelines [29, 30], where σ is the standard deviation of the intercept and S is the slope of the calibration graph, are used to determine both LOD and LOQ. LOD values for TIN and CIP were 6.7 and 25.03 ng/band, respectively, while LOQ values for TIN and CIP were 20.3 and 75.34 ng/band, respectively.

Table 6 Application of the proposed HPTLC method for the determination of TIN and CIP in tablet dosage form (**A**) and spiked human plasma (**B**)

A						B				
		% Recovery[a] ± SD		*t* Value[b]	*F* value[b]		TIN		CIP	
		Proposed	Reported[c]			Added conc	Found conc.[a]	% Recovery ± SD	Found conc.[a]	% Recovery ± SD
Tinifloxacin tablet	600 mg TIN/tablet	99.90 ± 0.46	99.10 ± 0.60	2.05	1.43	100	98.6	98.6 ± 0.46	98.8	98.8 ± 0.27
	500 mg CIP/tablet	99.45 ± 0.25	99.85 ± 0.81	2.10	1.65	250	247.5	99.0 ± 0.32	248.6	99.4 ± 0.35
						500	493.0	98.6 ± 0.47	492.4	98.5 ± 0.82

[a]The values are the mean of five determinations

[b]The tabulated *t* and *F* values at 95% confidence limit are 2.78 and 6.39, respectively

[c]Reported method [13]

3.3.2 Accuracy and precision

Within the analytical range of each of the investigated pharmaceuticals, the accuracy of the developed HPTLC technique was assessed at five concentration levels (150, 250, 400, 500, and 800 ng/band) (triple measurements of each concentration). The percentage of recovery and standard deviation of the measurements are displayed in Table 2. The findings show that the proposed approach is quite accurate.

The precision of the analytical procedure, on the other hand, was assessed by calculating both intra-day and inter-day precision. Three concentration levels of each medication were used to determine intra-day accuracy, and each concentration was reproduced three times on the same day (repeatability). Following development, the inter-day precision of each medication was assessed using three concentration levels; each concentration was repeated three times over three days, and each sample injection volume was repeated three times (intermediate precision). The findings (Table 3) show that the suggested analytical approach has good accuracy at both the inter-day and intra-day levels.

3.3.3 Method selectivity

For approval of the suggested analytical technique's selectivity, several laboratory-prepared mixtures of the studied pharmaceuticals were analyzed using the proposed analytical technique. The obtained data, given in Table 4, show that the examined medicines had a high percent recovery rate, indicating their selectivity [18, 31, 32]

3.3.4 Method robustness

To test the robustness of the suggested approach, it was utilized to analyses of the researched medicines using the proposed analytical methods but with a modest variation in the mobile phase composition. Any modification in the mobile phase composition results in a modest change in the R_F value of the investigated but no change in the area under peak, according to the findings (Table 5).

3.3.5 Application to pharmaceutical formulation

The suggested analytical approach was shown to be effective in analyzing the medicines investigated in their pharmaceutical dosage formulation. The method's selectivity was tested by looking for any effects from tablet excipients. The results show that tablet excipients did not cause any interference, confirming the accuracy of the suggested approach. The resulting findings were compared to those obtained using the previously described method [10]. In terms of precision and accuracy, Student's *t* test and *F* test are used. Table 6

shows that there was no significant difference in the results produced from both approaches, as demonstrated by Student's *t* test and *F* test, because the calculated values did not exceed the theoretical values at the 95% confidence level. This demonstrates the suggested method's great precision and accuracy.

3.3.6 Spiked human plasma

With excellent success, the proposed technique was employed to analyze the investigated medicines in spiking human plasma. The regression equation for each drug was used to determine its concentration. The standard solutions of the studied drugs were spiked into human plasma at concentrations of 100, 250, and 500 ng. The results are shown in Table 6. The mean percent of recoveries of the tested drugs concentration in plasma ranged from 98.5 to 99.4, with standard deviations ranging from 0.27 to 0.82, demonstrating that the pharmaceuticals studied may be identified properly and precisely in spiked human plasma without interference.

4 Conclusion

For the simultaneous determination of TIN and CIP, the suggested technique offers a high sensitivity, quick, simple, and green micellar HPTLC method. The suggested approach does not require any prior sample preparation for the extraction phase, which most analysts dislike; also, unlike existing HPLC procedures, the proposed method does not require any expensive chemicals. Because of its greenness and low cost, it is necessary to be utilized in the regular analysis of the examined pharmaceuticals in many quality-control laboratories, in addition to the ability to use the mobile phase several times with multiple plates.

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Declarations

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

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