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ORIGINAL RESEARCH PAPER

Smart TLC–densitometric methods for determination of ophthalmic ternary mixture containing chloramphenicol in the presence of its synthetic precursor: Comparative eco-scaling for greenness assessment

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Abstract
Green analytical methods have gained a growing interest in the field of pharmaceutical research to reduce impacts on the environment and enhance analysts' health safety. Chloramphenicol (CHL), dexamethasone sodium phosphate (DSP) and tetrahydrozoline HCl (THZ) form an ophthalmic ternary mixture that is co-formulated for conjunctivitis treatment. In the present work, for time saving and higher sensitivity, two green thin-layer chromatography (TLC) methods were developed for the determination of this ophthalmic ternary mixture in the absence or presence of p-nitroacetophenone (PNA), a synthetic precursor of chloramphenicol. In both proposed methods, silica gel 60 F_{254} plates were used as the stationary phase. The mobile phase used for method (A) was ethanol−water−ammonia (7.0:2.5:0.5, *V/V*), while, for method (B), acetonitrile−water−ammonia (10.0:3.0:0.5, *V/V*) was used as the mobile phase. TLC separation was followed by quantitative determination of the aforementioned drugs at wavelengths 242.0 nm and 220.0 nm. Both methods were validated in compliance with the International Conference on Harmonisation (ICH) guidelines, where both methods were found to be reliable, reproducible, and selective. Statistical comparison of the developed methods was done with a reported high-performance liquid chromatography (HPLC) method where no significant difference was found. Analytical eco-scaling depends on penalty point which was calculated to be 92, 88 and 87 for methods A, B and the reported HPLC, respectively, suggesting that the proposed methods are eco-friendlier with penalty point scoring very high on the scale than the reported one.

Keywords Eco-scaling · Thin-layer chromatography · Chloramphenicol · Dexamethasone sodium phosphate · Tetrahydrozoline HCl · Synthetic precursor

1 Introduction

In recent years, the use of green solvents to establish green analytical methodologies has considerably grown. In green analytical chemistry (GAC), the definition calls for a reduction or total removal of harmful chemicals used in the analytical process, a reduction in energy consumption and minimization of waste production, without compromising the requirements for optimum performance of a system [1–3]. Thin-layer chromatography (TLC) has emerged as a significant step towards improved separation efficiency, allowing faster analysis, shorter peaks, better resolution [4, 5]. A significant advantage of TLC over high-performance liquid chromatography (HPLC) is the ability to run multiple samples in parallel and allow 20 samples to be spotted, isolated and quantified simultaneously on a small plate (10 cm × 20 cm) [2], while the samples in HPLC should be quantitatively injected into a pre-washed and conditioned column, resulting in a delay in data acquisition [6, 7].

Conjunctivitis and keratitis are bacterial infections related to perceived health risks with severe eye pain, blurring of vision and extreme photosensitivity as major symptoms [8]. The most frequent drug classes used for curing conjunctivitis are antibacterial, anti-inflammatory and sympathomimetic

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drugs. Chloramphenicol (CHL) has a bacteriostatic action that is effective against Gram-negative and Gram-positive bacteria (Fig. 1a) [9]. CHL has benefits of being cheap and more readily available than other antibiotics [10]. Dexamethasone sodium phosphate (DSP) is a water-soluble and inorganic ester that has anti-inflammatory activity (Fig. 1b). DSP is often used to treat adrenal cortex insufficiency disorder [11, 12]. Tetrahydrozoline hydrochloride (THZ) has a sympathomimetic activity and is used as decongestant for conjunctiva (Fig. 1c) [13, 14]. p-Nitroacetophenone (PNA) is a synthetic precursor of CHL; it is harmful to the eye and causes serious eye irritation if it is present in an eye drop (Fig. 1d) [15, 16]. Ocuphenicol-D® eye drop is available in the market, contains the three aforementioned drugs and is recommended for acute and chronic infectious conjunctivitis.

Reviewing the literature, various spectrophotometric methods were used to evaluate this mixture in its pharmaceutical dosage form [17, 18]. Three HPLC chromatographic methods for evaluating this mixture have also been reported [19–21]. Also a voltammetric method was mentioned [22]. The majority of the reviewed methods highly experienced either pre-separation or time-consuming excessive data treatment. To the best of our knowledge, no TLC–densitometric methods have been developed for the simultaneous determination of the studied ternary mixture in pharmaceutical preparation until now.

According to this, the present work introduces first, green, selective and sensitive TLC–densitometric methods which are time-saving for the determination of ophthalmic ternary mixture either in absence or presence of PNA by using GAC. The developed TLC methods have been compared to the reported HPLC method regarding eco-scaling for green assessment [21]. Eco-scaling of TLC methods was calculated depending on penalty points (based on reagents and instruments) and subtracted from a base of 100 (the score of an ideal green analytical method) [23]. Statistical comparison of the developed methods was done with the reported HPLC method where no significant difference was found [21].

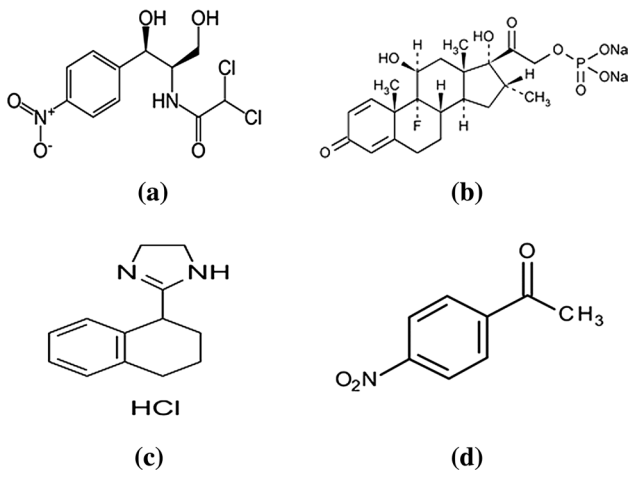

Fig. 1 The chemical structures of **a** chloramphenicol, **b** dexamethasone sodium phosphate, **c** tetrahydrozoline hydrochloride and **d** p-nitroacetophenone

2 Experimental

2.1 Materials and reagents

2.1.1 Pure samples

CHL and DSP were kindly provided by the Egyptian International Pharmaceutical Industries Co. (EIPICO; Cairo, Egypt), while THZ was supplied by Orchidia Company (Cairo, Egypt). PNA was bought from Sigma-Aldrich (Cairo, Egypt). Their purity was found to be 99.47% for CHL, 99.36% for DSP, 100.63% for THZ and 98% for PNA, according to the official methods [24].

2.1.2 Pharmaceutical formulation

Ocuphenicol-D® eye drop (Batch No. 8529007) is claimed to have 5.0 mg of CHL, 1.0 mg of DSP and 0.25 mg of THZ per mL, which are produced by Alexandria Co. for Pharmaceuticals and Chemical Industries (Alexandria, Egypt).

2.2 Chemicals and solvents

All the chemicals and solvents used were of analytical grade and were used without further purification. Methanol, acetonitrile, ethanol and ammonia 30% (Merck, Darmstadt, Germany), ultra-pure water 18.2 MΩcm (Adwic, Cairo, Egypt) were used.

2.3 Standard solutions

Stock standard solutions of CHL, DSP and PNA (1.0 mg/mL) were separately prepared by using methanol as solvent by the two proposed TLC methods, while stock standard solutions of THZ were prepared in method (A) as 5.0 mg/mL and in method (B) as 1.0 mg/mL, using the same solvent. Working standard solutions of CHL, DSP and PNA (100.0 µg/mL) were separately diluted from the stock standard solutions by using methanol as the diluent, while working standard solutions of THZ were prepared by dilution from the stock standard solutions to obtain concentrations of 500.0 µg/mL in method (A) and 100.0 µg/mL in method (B).

2.4 Apparatus and software

The following were used: TLC densitometer (CAMAG, Muttenz, Switzerland) Linomat 5 autosampler supplied with a 100 µL CAMAG micro-syringe; a Model 3 densitometer CAMAG TLC Scanner 3 supplied with winCATS software; precoated TLC sheets; silica gel 60 F_{254} (20 cm × 20 cm) plates (Merck).

2.5 Chromatographic conditions

Chromatographic separation was carried out on TLC aluminum sheet coated with silica gel 60 F_{254} (Merck) plates (10 cm × 20 cm) as the stationary phase. For method (A), the mobile phase used consisted of ethanol−water−ammonia (7.0:2.5:0.5, *V/V*), while, for method (B), acetonitrile−water−ammonia (10.0:3.0:0.5, *V/V*) was used as the mobile phase. In the two methods, the studied drugs' solutions were applied as separate compact spots 15 mm from the bottom of the plates, with a 3 mm band width and with slide dimensions 6.0 mm × 0.3 mm and scanning rate of 20 mm/s. In the beginning, TLC plates were activated at 100 °C for 20 min to remove any moisture [25]. Saturation of chromatographic tank was done with each mobile phase separately for 30 min prior to development. The normal-phase TLC plates were developed over 8 cm in an ascending manner, then they were left to dry in air and then scanned specifically at 220.0 nm for THZ, while for the other drugs at 242.0 nm in both methods.

3 Procedure

3.1 Construction of calibration curve

Constant volumes of different concentrations of each drug were spotted on TLC plates by using the CAMAG Linomat auto-sampler with a micro-syringe (100 µL), then analyzed under the previously mentioned chromatographic conditions described for each method. The different concentrations were 0.1−1.4 µg/band for CHL, 0.2−1.2 µg/band for DSP and 0.1−0.5 µg/band for THZ in method (A), while 0.6−1.8 µg/band for CHL, 0.8−3.2 µg/band for DSP, 0.32−1.4 µg/band for PNA and 0.01−0.1 µg/band for THZ in method (B). The calibration curves were constructed by plotting the corresponding concentrations *versus* the mean integrated peak area and then the regression equations were computed.

3.2 Analysis of pharmaceutical preparation

Ocuphenicol-D® eye drop is claimed to have 5.0 mg of CHL, 1.0 mg of DSP and 0.25 mg of THZ per mL. 1 mL from dosage form was transferred into a 10-mL volumetric flask to reach concentrations of 500.0 µg/mL for CHL, 100.0 µg/mL for DSP and 25.0 µg/mL for THZ. The concentration of each drug was calculated from the corresponding regression equation.

4 Results and discussion

Zero order absorption spectra of 18 µg/mL each of CHL, DSP, THZ and PNA were scanned from 200 to 400 nm using methanol as the blank (Fig. 2), showing severe overlapping that hinders direct spectrophotometric determination of the studied components.

With regard to optimization of the proposed methods, we have kept in mind that effort should be made to achieve an eco-friendly solvent system without diminishing the analytical performance. The design of the method in planar chromatography requires two crucial steps to obtain adequate qualitative and quantitative analytical results. The first step is the optimization of the composition of the mobile phase; the second one is the stationary phase.

The most tedious step in the development of the TLC method is generally to find the optimal solvent system. Although non-polar solvents such as chloroform, benzene and toluene are commonly used in developing systems, these solvents are excluded from our trials due to their known environmental toxicity. Several experiments were performed using different mobile phase systems with different ratios and compositions such as water−ethyl acetate−ammonia, but no satisfactory separation was obtained. Other systems as butanol−water−acetic acid were tried, but did not improve the separation.

Finally, separation of CHL, DSP, THZ was obtained by using ethanol−water−ammonia (7.0:2.5:0.5, *V/V*) as developing system in method (A). A satisfied separation for CHL, DSP, THZ and PNA was obtained by using

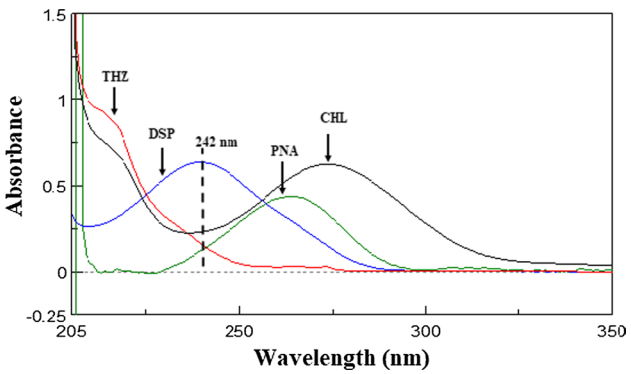

Fig. 2 UV absorption spectra of CHL, DSP, THZ and PNA using methanol as solvent

acetonitrile−water−ammonia (10.0:3.0:0.5, *V*/*V*) in method (B). Although method (A) is proved to be greener than method (B), it failed to separate PNA from CHL thus we searched for another mobile phase that could separate the four components as described in method (B).

The polarity of mobile phase was alerted upon replacing ethanol (polarity index 5.2) by acetonitrile (polarity index 5.8) and, as it is known that "like dissolve like" [26], in method (A), THZ—being less polar than DSP due to the presence of one hydrogen bond acceptor and two hydrogen bonds donor—was eluted first, while, in method (B), DSP was eluted first as it is the most polar compound in the mixture due to the presence of six hydrogen bonds acceptor and three hydrogen bonds donor. Two band widths were tested as 3 mm and 6 mm and the best results were obtained using 3 mm.

In method (A), densitometric TLC separation was performed at 242.0 nm and the obtained R_F values were 0.20 ± 0.02, 0.65 ± 0.02, 0.76 ± 0.02 for THZ, DSP and CHL, respectively (Figs. 3a, 4a). In method (B), densitometric

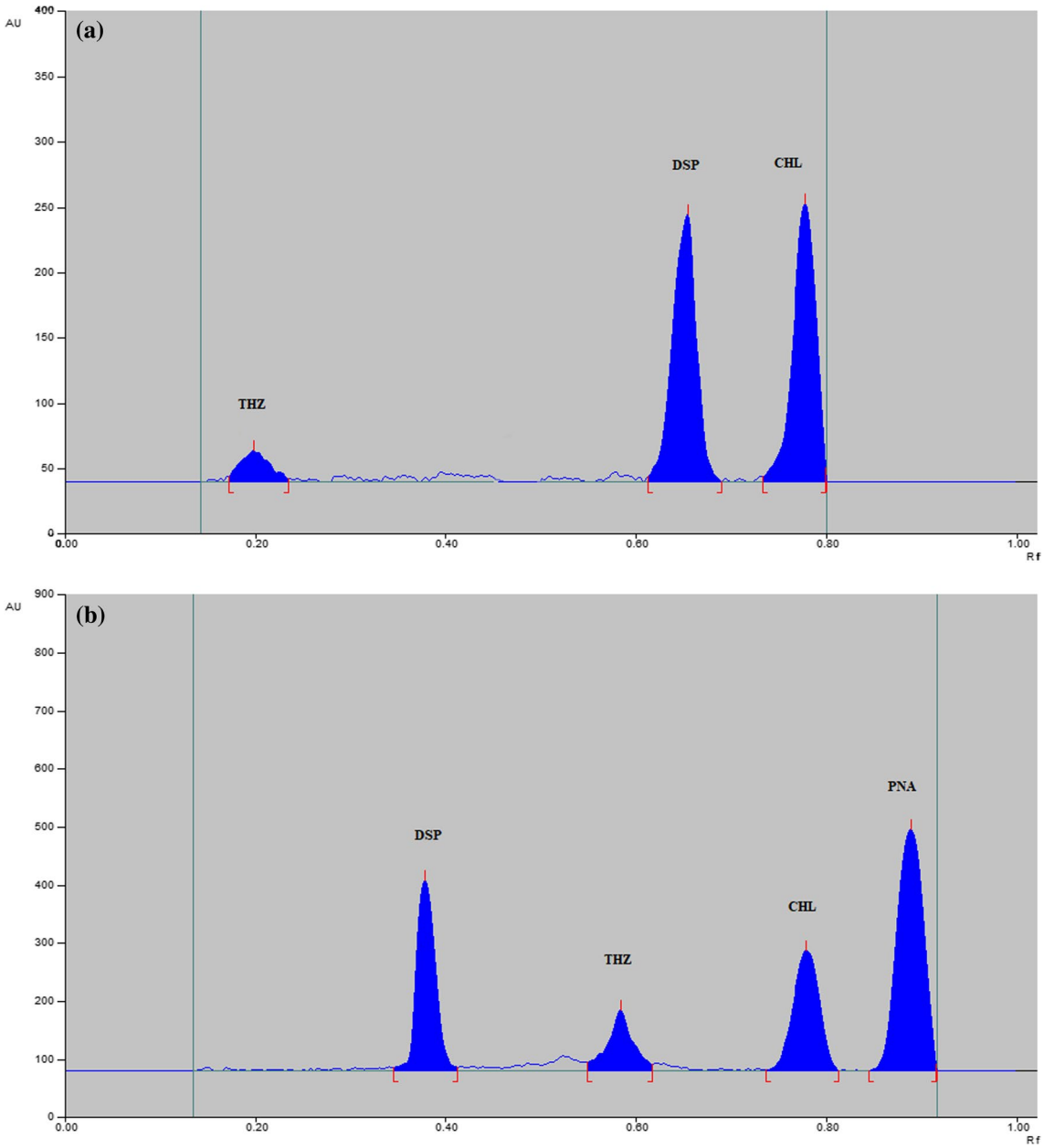

Fig. 3 **a** 2D TLC densitogram of method (A) of separated peaks of THZ ($R_F = 0.20 \pm 0.02$), DSP ($R_F = 0.65 \pm 0.02$) and CHL ($R_F = 0.76 \pm 0.02$). **b** 2D TLC densitogram of method (B) of separated peaks of DSP ($R_F = 0.38 \pm 0.02$), THZ ($R_F = 0.58 \pm 0.02$), CHL ($R_F = 0.78 \pm 0.02$) resolved from its synthetic precursor PNA ($R_F = 0.87 \pm 0.02$)

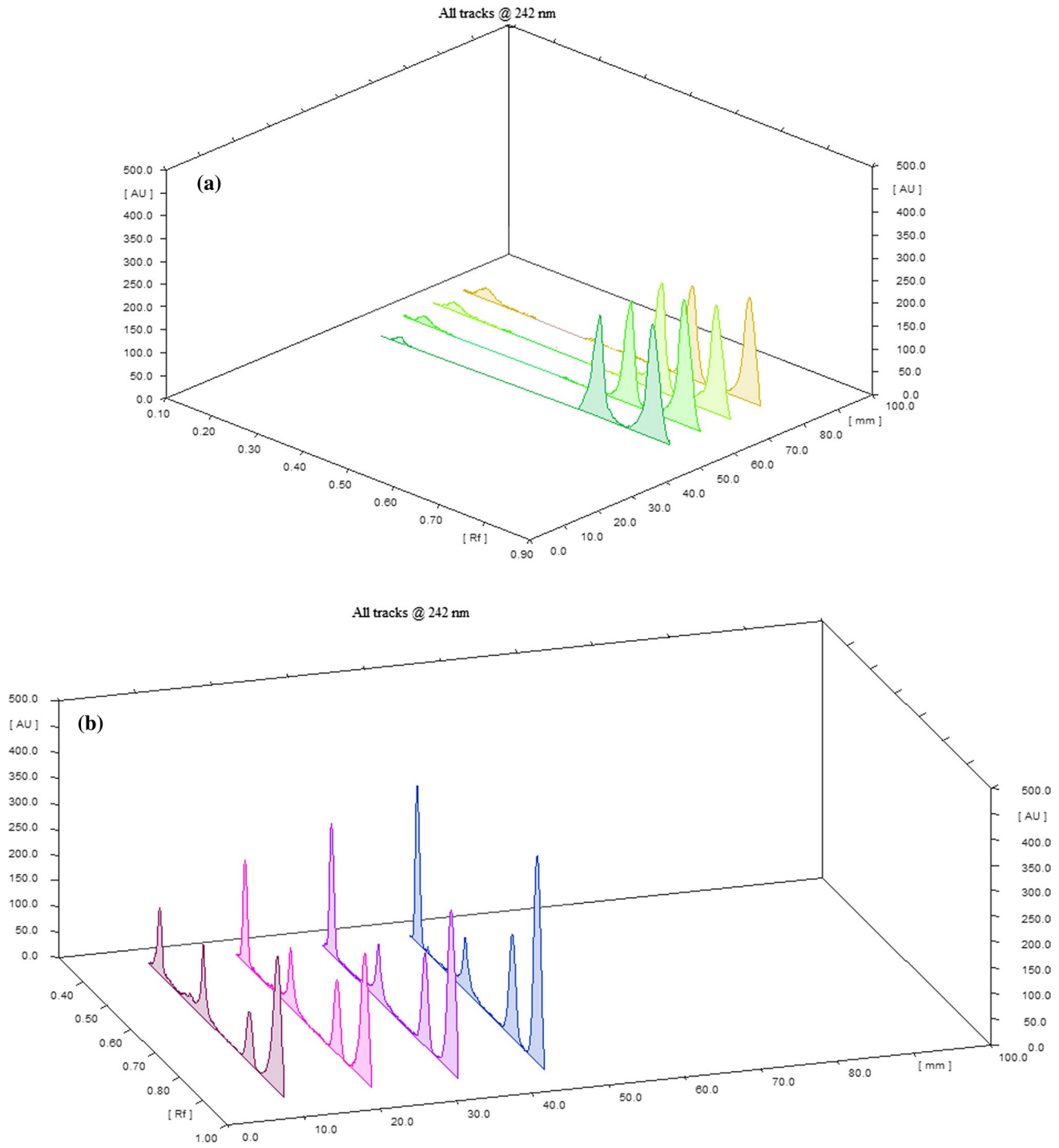

Fig. 4 **a** 3D TLC chromatograms of method (A) of laboratory prepared mixtures at 242.0 nm. **b** 3D TLC chromatograms of method (B) of laboratory prepared mixtures at 242.0 nm

TLC separation was performed at 242.0 nm and the obtained R_F values were 0.38 ± 0.02, 0.58 ± 0.02, 0.78 ± 0.02 and 0.87 ± 0.02 for DSP, THZ, CHL and PNA, respectively (Figs. 3b, 4b).

4.1 Scanning wavelength

The UV spectra of the studied components are shown in Fig. 2. Different wavelengths were tried to compromise

between the highest sensitivity for CHL, DSP, THZ and PNA and the lowest noise, including 220.0, 242.0, 254.0, and 275.0 nm. The best scanning wavelength was 242.0 nm as shown in Figs. 3, 4) presenting sharp, untailed, and well-separated peaks.

4.2 Validation parameters

The proposed methods were validated according to the International Conference on Harmonisation (ICH) guidelines [27]. In both methods, calibration was computed by relating the obtained peak areas at 242.0 nm to the corresponding concentrations of drugs and the relations were linear as in CHL and DSP over the ranges of (0.1−1.4 µg/band) and (0.2−1.2 µg/band), respectively, in method (A), while they were (0.6−1.8 µg/band) and (0.8−3.2 µg/band) in method (B), respectively. THZ was specifically calibrated at 220.0 nm in both methods because it was the most sensitive wavelength in the range (0.1−0.5 µg/band) in method (A) and (0.01−0.1 µg/band) in method (B), while PNA was calibrated at 242.0 nm in the range (0.32−1.4 µg/band) in method (B) only. The proposed methods were validated regarding linearity, range, accuracy, precision, limit of

Table 1 Validation parameters of the developed TLC–denstiometric methods for the determination of CHL, DSP, THZ and PNA

Parameters	Method A			Method B			
	CHL (242.0 nm)	DSP	THZ (220.0 nm)	CHL (242.0 nm)	DSP	PNA	THZ (220.0 nm)
Range [µg/band] linearity	0.1−1.4	0.2−1.2	0.1−0.5	0.6−1.8	0.8−3.2	0.32−1.4	0.01−0.1
Correlation coefficient [*r*]	0.9997	0.9995	0.9995	0.9996	0.9993	0.9992	0.9996
Slope	1917.42	3476.81	3502.1	3736.05	1611.24	5314.87	8146.96
Intercept	4138.36	1174.98	56.59	741.40	2597.35	10,336.77	847.23
Accuracy[a] [mean% ± SD]	100.09 ± 0.62	100.22 ± 1.22	100.56 ± 0.88	100.05 ± 0.16	99.92 ± 1.17	–	100.24 ± 1.46
Precision[b] (*%RSD*)							
Repeatability	0.77	1.07	0.73	0.32	0.85	–	1.02
Intermediate	1.24	1.32	0.92	0.74	1.35	–	1.57
LOD[c] [µg/band]	0.03	0.04	0.02	0.06	0.13	0.07	0.003
LOQ[c] [µg/band]	0.09	0.12	0.06	0.18	0.39	0.21	0.009

[a]Average of three different concentrations repeated three times within the day

[b]Precision was evaluated by measuring the response of three concentrations of each drug three separate times on the same day (repeatability) and on three different days (intermediate) precision in method A, CHL (0.9, 1.5 and 1.7 µg/band), DSP (1.6, 2.0 and 3.0 µg/band) and THZ (0.02, 0.06 and 0.08 µg/band). While in method B another different concentration was used such as (0.3, 0.6 and 1.3 µg/band) for CHL, (0.3, 0.9 and 1.1 µg/band) for DSP and (0.15, 0.35 and 0.45 µg/band) for THZ

[c]LOD and LOQ were calculated from the standard deviation (*s*) of the response and the slope of the calibration curve (*S*) according to the following equations: LOD = 3.3 (*s*/*S*) and LOQ = 10 (*s*/*S*)

Table 2 System suitability parameters of the developed TLC–denstiometric methods for the determination of CHL, DSP, THZ and PNA

Parameters	Method A			Method B				Reference value [27]
	THZ	DSP	CHL	DSP	THZ	CHL	PNA	
Retention factor (R_F)	0.20	0.65	0.76	0.38	0.58	0.78	0.87	–
Resolution (R_s)	15.07	3.23		8.07	5.87	3.07		> 2
Capacity factor (*K*)	4.0	0.53	0.31	1.63	0.72	0.28	0.14	0–10
Selectivity (α)	7.42	1.70		2.15	2.56	1.88		> 1
Tailing factor (*T*)	1.03	0.95	0.96	1.01	0.97	0.97	0.96	*T* = 1 for a symmetric peak

Table 3 Robustness assessment of the adopted TLC–densitometric methods for determination of CHL, DSP and THZ

Parameters	Measured R_F				Measured K				Measured T			
	Saturation time (min)		Mobile phase composition (mL)[a]		Saturation time (min)		Mobile phase composition (mL)[a]		Saturation time (min)		Mobile phase composition (mL)[a]	
Method A	30 − 5	30 + 5	7 − 0.5[a]	7 + 0.5[a]	30 − 5	30 + 5	7 − 0.5[a]	7 + 0.5[a]	30 − 5	30 + 5	7 − 0.5[a]	7 + 0.5[a]
Method B			10 − 0.5[b]	10 + 0.5[b]			10 − 0.5[b]	10 + 0.5[b]			10 − 0.5[b]	10 + 0.5[b]
CHL												
Method A	0.77	0.76	0.76	0.77	0.33	0.27	0.35	0.33	0.95	0.96	0.96	0.95
Method B	0.79	0.78	0.78	0.79	0.30	0.26	0.32	0.30	0.96	0.97	0.97	0.96
DSP												
Method A	0.66	0.65	0.65	0.66	0.56	0.49	0.59	0.55	0.94	0.95	0.95	0.94
Method B	0.39	0.38	0.38	0.39	1.65	1.59	1.70	1.67	1	1.01	1	1.01
THZ												
Method A	0.21	0.20	0.20	0.21	4.3	3.8	4.5	4.2	1.04	1.03	1.03	1.04
Method B	0.59	0.58	0.58	0.59	0.76	0.68	0.79	0.75	0.98	0.97	0.97	0.98

[a]Change in ethanol content

[b]Change in acetonitrile content

detection and limit of quantification according to ICH guidelines [27], presented in Table 1.

System suitability parameters for the proposed TLC methods were calculated and satisfactory results were obtained as summarized in Table 2. Robustness was tested by checking little changes occurring in R_F, K and T upon changing saturation time and the mobile phase composition. The results illustrate the robustness of the suggested methods as shown in Table 3.

4.3 Analytical eco-scale greenness evaluation of the proposed TLC methods versus the HPLC method

Evaluating the environmental impact of the different analytical approaches with respect to their conformity to the principle of green chemistry was very important, away from personal impressions or uncertain assumptions. In this sense, several GAC assessment methods (green metrics) are implemented to check the greenness of each analytical method, quantitatively or qualitatively [28]. Eco-scale analysis is a semi-quantitative ecological metric method used to test analytical procedures, thus the comparison and selection of the greenest alternative can be achieved [29]. The eco-scale tool is dependent on penalty point from a base of 100 (the perfect green analytical method score). Penalty points are allocated and subtracted from 100 for each of the analytical process parameters (quantity and nature of reagents, occupational hazard, energy consumed and waste generated) [30]. The higher the score, the greener and the more economical is the analytical process. The result of the calculations is ranked on a scale where the score > 75 refers to a great green analysis, between 75 and 50 to an acceptable green analysis and < 50 to an inadequate green analysis [31]. The analytical eco-scale for the developed TLC methods and the reported HPLC method was calculated, and the results demonstrated that the proposed TLC methods excel over HPLC as a greener alternative for the simultaneous analysis of CHL, DSP and THZ. Method (A) was more greener than method (B) as shown in Table 4 [32].

4.4 Statistical comparison

Both Student's *F*-test and *t*-test were conducted; the proposed methods for analyzing the ternary mixture in pharmaceutical preparation were successfully applied using two mobile phases and were statistically compared with the recorded HPLC process as summarized in Table 5 [21]. No significant statistical difference was observed between the proposed and the reported methods.

5 Conclusion

The emergence of the green chemistry concept has driven researchers and chemists in all fields to recognize the environmental impact of their chemicals used in their methods and to determine the greenness of their processes. In this context, green and validated two TLC–densitometeric methods were developed to determine a ternary mixture of CHL, DSP and THZ in pure and medicinal forms even without a synthetic precursor of CHL. The TLC methods

Table 4 Penalty points (PPs) for the two proposed TLC methods and the reported HPLC method

Parameters	Penalty points [PPs]		
	Method A	Method B	Reported method [21]
Reagents			
Water	0.0	0.0	–
Ethanol	0.0	–	–
Ammonia	2.0	2.0	–
Acetonitrile	–	4.0	4.0
Phosphate buffer	–	–	0.0
Instrument			
Energy [˃0.1 kWh per sample]	1.0	1.0	1.0
Occupational hazard	0.0	0.0	3.0
Waste	5.0	5.0	5.0
Total PPs	Σ8	Σ12.0	Σ13.0
Analytical eco-scale score	92.0 Excellent green analysis	88.0 Excellent green analysis	87.0 Excellent green analysis

Table 5 Statistical analysis of the results obtained by the proposed TLC methods and the reported HPLC method for the determination of CHL, DSP, THZ in pharmaceutical preparation

Parameters	CHL			DSP			THZ		
	Method A	Method B	Reported method[a]	Method A	Method B	Reported method[a]	Method A	Method B	Reported method[a]
Mean[b] [%]	96.83	99.38	100.25	100.74	100.63	101.33	97.44	98.74	99.97
SD	0.74	0.75	0.30	1.06	0.64	1.66	1.08	0.96	0.89
Variance	0.56	0.57	0.09	1.14	0.41	2.77	1.17	0.93	0.80
N	3	3	3	3	3	3	3	3	3
Student's *t*-test[c] (2.78)	0.14	0.54	–	1.94	1.46	–	0.32	0.62	–
F-value[c] (19.0)	6.24	6.37	–	2.43	6.62	–	1.47	1.17	–

[a]HPLC method: using C_{18} column and mobile phase consisting of acetonitrile–phosphate buffer (30:70, *V/V*) at a flow rate of 1.0 mL/min and detection at 230.0 nm [21]

[b]Average of 3 experiments

[c]Figures between parentheses represent the corresponding tabulated values of *t* and *F* at $p = 0.05$

provide shorter analytical time, lower detection and quantification limits, lower mobile phase and enhanced resolution. Eco-scale was calculated for the suggested TLC methods and the reported method, taking into account the use and quantity of reagents, the use of instruments, the energy consumed and the waste produced, and the suggested methods proved to be more environmental-friendly, scoring very high on the scale, with good performance and validation parameters. The proposed methods could, therefore, be a convenient alternative for the routine analysis of the pharmaceutical mixture being studied in a safer manner, particularly in those laboratories that lack more an advanced instrument. Statistical comparison between the two proposed methods and the reported method exposed no pronounced difference, which proved their sensitivity, accuracy and precision. However, the suggested methods are more suitable and less problematic than the reported one for the determination of the studied mixture of drugs. The proposed methods were validated according to ICH guidelines to be used for the determination CHL, DSP and THZ with highly accurate and precise results.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest to declare.

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