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Application and validation of an eco-friendly TLC-densitometric method for simultaneous determination of co-formulated antihypertensive agents

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Owing to the greater awareness that has arisen within the analytical community regarding the colossal negative influence of hazardous chemicals on both health and the environment, there is an increasing interest in developing more environment-friendly practices in different research areas. In this context, an eco-friendly TLC-densitometric method was designed, optimized and validated for the quantitative analysis of a ternary pharmaceutical mixture used for hypertension management, in the pure powdered form, synthetic mixtures and combined pharmaceutical formulation. The proposed method is based on the separation of the three co-formulated drugs; telmisartan (TL), hydrochlorothiazide (HZ) and amlodipine besylate (AM) on silica gel F_{254} plates, using green solvents as the developing system. The method was validated with regard to linearity, accuracy, precision, system suitability, and robustness. Comparison of the suggested method to the reported conventional TLC-densitometric method, regarding their validation parameters and greenness profiles, was carried out. The suggested method was found to be more eco-friendly and more solvent-/time-saving; hence it can be used for quality control analysis of the studied mixture in a safer way.

1. Introduction

Many trials are being carried out and continuous efforts are being made to diminish the injurious impacts of hazardous chemicals on humans and the environment. As a result, implementation of the aspects and principles of Green Analytical Chemistry (GAC) have increased.[1,2] The green analytical chemistry concept signifies the diminishment or elimination of hazardous solvents and chemicals from analytical processes to result in an environment-friendly analysis without conceding the performance criteria of the analytical method.

Several strategies have been studied and applied to reduce the harmful impact of different analytical methodologies. Special consideration has been given to the use of hazardous solvents,[3] which is considered the biggest challenge for greening analytical methods, particularly the chromatographic ones.[4,5] Either by decreasing contact with a definite hazardous solvent or omitting the different noxious solvents and reagents, researchers can increase the greenness profile of the different analytical procedures. Full exclusion of specific solvents and reagents is not always feasible without a significant waning of the analytical results. In these cases, it is sensible and highly recommended to swap the harmful solvents and reagents for greener alternatives. Alternatively, if it is not feasible to substitute the hazardous reagents with alternatives that are more environment-friendly, their minimization would be a viable possibility, without reducing the effectiveness of the applied method.

Thin-layer chromatography (TLC) is a powerful method equally appropriate for both qualitative and quantitative analytical tasks. Many applications of TLC-densitometric methods for identification and quantitation of impurities and impurity level targets, constituents, active substances and degradation products, and also for kinetics studies, process development and optimization, process monitoring, and cleaning validation, have been demonstrated.[6–8] It is superior to other analytical techniques in terms of total cost and time for analysis as it doesn't require tedious cleanup; with the use of appropriate mobile phases and reagents, all interfering agents will be omitted. It is a very rapid, accurate and precise chromatographic technique for different components assays and can be used for routine quality control of pharmaceutical products. By eliminating and/or minimizing the use of hazardous solvents, TLC methods could be modified to be more eco-friendly.

A co-formulated fixed dose pill composed of telmisartan (TL) (an angiotensin II antagonist),[9] hydrochlorothiazide (HZ) (a thiazide diuretic that lowers blood pressure and is used in combination therapy with other antihypertensive agents)[10] and

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amlodipine besylate (AM) (a calcium channel blocker, used for stable angina pectoris and adjunct therapy for hypertension),[11] Fig. 1, has been recently used for hypertension management as a triple-combination therapy. It proved to have the potential for quicker lowering of blood pressure, obtaining target blood pressure, and minimized adverse effects.[12,13]

To our knowledge, very few methods have been reported for simultaneous determination of this ternary mixture; they include UPLC[14] and RP-HPLC and HPTLC densitometric[15] methods. The reported densitometric method used chloroform : methanol : formic acid (85 : 15 : 5 by volume) as the developing system and the R_f values were 0.73, 0.41 and 0.31 for TL, AM and HZ; respectively.

The goals of our study are, firstly, to develop and validate a green TLC densitometric method for the simultaneous determination and quality control analysis of telmisartan (TL), hydrochlorothiazide (HZ) and amlodipine besylate (AM) in their ternary mixture; secondly, to validate the proposed method and statistically compare the analysis results to those of the cited drugs' official methods; and finally, to compare the proposed method to the reported conventional TLC-densitometric method regarding their validation parameters and greenness profiles. The suggested method was found to be more eco-friendly and more solvent-/time-saving without affecting the analytical and validation parameters of the method, as proved by the study results.

2. Experimental

2.1. Instruments

The plates used were 20 × 20 cm, coated with 0.25 mm silica gel 60 F254 (Merck, Darmstadt, Germany). The samples were applied to the plates using a Camag Linomat 5 autosampler (Camag, Muttenz, Switzerland) with a Camag micro-syringe (100 μL). A Camag TLC scanner model 3S/N 1302139 with winCATS software for densitometric evaluation (Camag, Muttenz, Switzerland) was used for scanning.

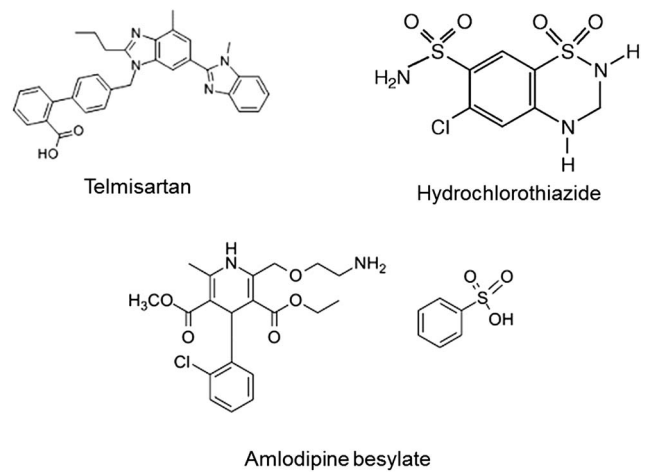

Fig. 1 Chemical structures of telmisartan, hydrochlorothiazide and amlodipine besylate.

2.1.1. Materials and reagents

Reference samples. The standard sample of TL was generously supplied by the National Organization of Drug Control and Research (NODCAR), Cairo, Egypt and HZ and AM standards were generously supplied by Al-Hekma Pharmaceuticals, Cairo, Egypt. The standards, TL, HZ and AM, have claimed purities of 99.89%, 100.45% and 99.93%, respectively, according to the official methods.[16]

Pharmaceutical formulations. Telma-AMH 40 tablets – batch number FT0114029. Each tablet is claimed to contain 40 mg of TL, 12.5 mg of HZ and 5 mg AM. Manufactured by: Glenmark Pharmaceuticals LTD, Mumbai, India.

Chemicals and reagents. All solvents and chemicals used were of analytical grade; ethyl acetate, methanol and acetone were purchased from Prolabo (VWR, International, West Chester, PA, USA).

2.2. Standard solutions

Stock solutions of TL, HZ and AM (1 mg mL^{-1}), where 0.05 g of each drug was accurately transferred into three separate 50 mL volumetric flasks, was dissolved in and diluted to volume with methanol.

3. Method

3.1. Chromatographic conditions

Firstly, the plates were washed and developed with the mobile phase by mixing ethyl acetate : methanol : acetone (7.5 : 2.5 : 0.5 by volume), then activated for 15 minutes by placing them in an oven at 100 °C prior to use. The three studied drug solutions were applied as separate compact spots 15 mm from the bottom of the plates, with 2 mm band length, and 150 nL s^{-1} dosage speed. The chromatographic tank was saturated with the mobile phase for 20 min. The plates were developed over a distance of 8 cm in an ascending manner then air-dried, and scanned under the following conditions: a deuterium lamp as the source of radiation, absorbance mode as the scan mode, slit dimensions of 3 mm × 0.45 mm, scanning speed of 20 mm s^{-1}, output as a chromatogram and integrated peak area, and measurements at 254 nm. Good separation of the three bands was shown by the difference in the retention factor (R_f) values: AM – $R_f = 0.1 \pm 0.01$, TL – $R_f = 0.39 \pm 0.01$ and HZ – $R_f = 0.82 \pm 0.01$, as shown in Fig. 2 and 3.

3.2. Linearity and construction of calibration graphs

Aliquots of TL (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 μL), HZ (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 μL) and AM (0.5, 1.5, 2.5, 3.5, 4.5 and 5.5 μL) standard solutions (1 mg mL^{-1}) were spotted on TLC plates, using the Camag Linomat autosampler with a micro-syringe (100 μL), and analyzed as per the previously mentioned chromatographic conditions. The calibration graphs of TL, HZ and AM were constructed by plotting the mean integrated peak area $\times 10^{-4}$ *versus* the corresponding concentration and then the regression equations were computed.

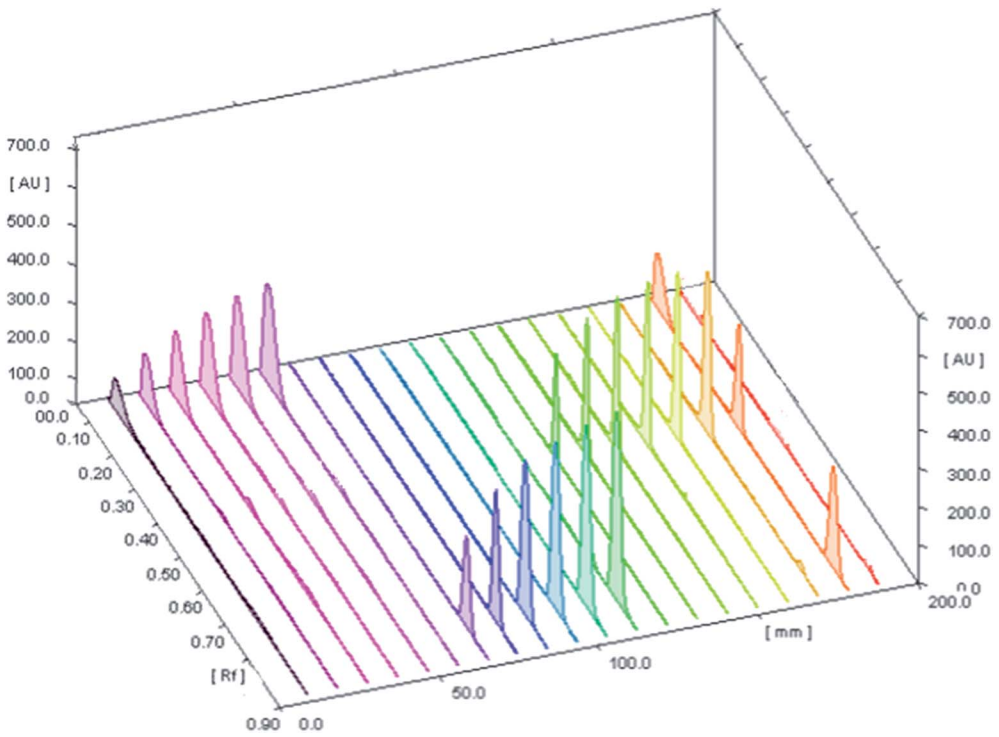

Fig. 2 TLC densitogram of AM ($R_f = 0.1 \pm 0.01$) in the concentration range 0.5–5.5 μg per band, TL ($R_f = 0.39 \pm 0.01$) in the concentration range 1.0–6.0 μg per band, HZ ($R_f = 0.82 \pm 0.01$) in the concentration range 0.5–3.0 μg per band and a mixture of TL, HZ and AM, using ethyl acetate : methanol : acetone (7.5 : 2.5 : 0.5 by volume) as a developing system, measured at 254 nm.

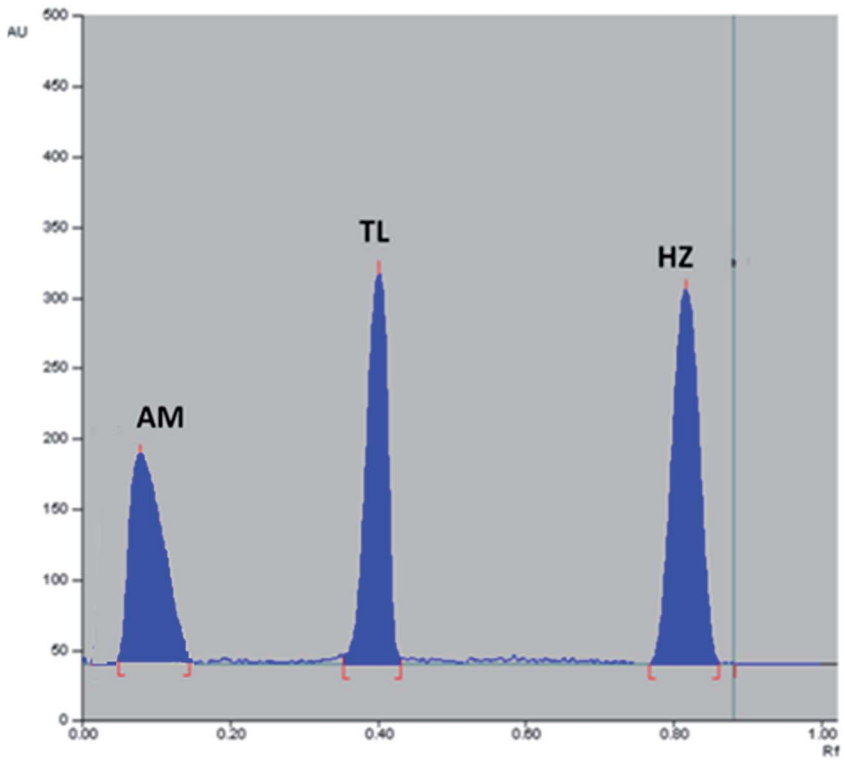

Fig. 3 2D TLC densitogram of separated peaks of AM ($R_f = 0.1 \pm 0.01$), TL ($R_f = 0.39 \pm 0.01$) and HZ ($R_f = 0.82 \pm 0.01$), using ethyl acetate : methanol : acetone (7.5 : 2.5 : 0.5 by volume) as a developing system, measured at 254 nm.

Table 1 Linearity studies and characteristic regression parameters for the proposed TLC-densitometric method

Parameter	TL	HZ	AM
Calibration range (μg per band)	1.0–6.0	0.5–3.0	0.5–5.5
Linearity			
Slope	0.5937	0.4502	0.2636
Intercept	0.174	0.098	0.0186
Correlation coefficient	0.9997	0.9994	0.9996
Mean ± % RSD	100.45 ± 0.836	99.96 ± 0.490	99.60 ± 0.682
Accuracy ± % RSD	100.02 ± 0.939	99.72 ± 0.791	100.11 ± 1.186
LOD (μg per band)	0.33	0.16	0.16
LOQ (μg per band)	1.00	0.48	0.48
Precision			
Repeatability *% RSD[a]	0.641	0.834	0.774
Intermediate precision *% RSD[b]	0.893	0.992	0.805

[a] *% RSD: the intra-day relative standard deviation, respectively ($n = 3$). [b] *% RSD: the inter-day relative standard deviation, respectively ($n = 3$).

3.3. Preparation and analysis of synthetic mixtures

Into a series of 10 mL volumetric flasks, aliquots of TL, HZ and AM standard solutions (1 mg mL^{-1}) were each accurately transferred to prepare mixtures containing different ratios of the three drugs. Aliquots of 10 μL from the prepared mixtures were spotted on TLC plates and the procedure was followed as described in the "Linearity and construction of calibration graphs" section; the concentrations were calculated from the corresponding regression equations.

3.4. Assay of pharmaceutical formulation

Ten tablets of Telma-AMH 40 were finely powdered. An amount of the powdered tablets equivalent to 100 mg TL was accurately weighed and transferred into a 100 mL beaker, then 20 mL methanol was added and the mixture was sonicated for 15 minutes and then filtered into a 100 mL measuring flask. The residue was washed (3 × 10 mL methanol) and the volume was completed up to the mark with methanol. Aliquots of 4 μL from the filtrate were spotted and analyzed as detailed in the "Linearity and construction of calibration graphs" section; the concentrations were calculated using the corresponding regression equations for TL, HZ and AM.

4. Results and discussion

One of the green chemistry principles is to promote the idea of "environment-friendly" (non-toxic) solvent replacement.[17–20]

Chloroform is a chlorinated solvent like carbon tetrachloride in its toxicity and is currently on the list of suspected carcinogens;[21,22] it is highly recommended to minimize its usage and replace it with a more environment-friendly substitute. The foremost challenge in applying the solvent replacement strategy is to increase the greenness of the method without affecting its validation parameters and effectiveness. In this context, studying the effect of different parameters to obtain maximum resolution is highly essential.

4.1. Optimization of chromatographic conditions

4.1.1. Developing system. In order to obtain the optimum separation, different solvent systems of variable compositions and ratios were tried, which were previously used for similar binary and ternary mixtures, avoiding the mostly used hazardous solvents like chloroform, benzene derivatives and others. In all the trials, the three drugs were applied on TLC plates, developed over a distance of 8 cm in an ascending manner using the different developing systems, then air-dried and scanned. The systems tried included a two-component

Table 2 Determination of telmisartan, hydrochlorothiazide and amlodipine besylate in their synthetic mixture using the proposed method

Concentration (μg per band) TL : HZ : AM	TL	HZ	AM
4 : 1.25 : 0.5	100.75	99.82	101.24
2 : 2: 2	100.31	98.65	100.72
2 : 2: 1	99.65	100.22	101.83
1.5 : 1.5 : 3	101.07	100.96	102.01
3 : 1.5 : 3	99.13	98.65	100.32
Mean ± % RSD	100.18 ± 0.791	99.66 ± 1.011	101.22 ± 0.717

Table 3 Robustness of the proposed TLC-densitometric method for the determination of TL, HZ and AM

	TL		HZ		AM	
Parameter	RSD% of peak area	$R_f \pm SD$	RSD% of peak area	$R_f \pm SD$	RSD% of peak area	$R_f \pm SD$
Mobile phase composition [ethyl acetate : methanol : acetone] (7.5 : 2.5 : 0.5, 7.7 : 2.3 : 0.5, 7.3 : 2.7 : 0.5)	0.653	0.39 ± 0.03	0.892	0.82 ± 0.03	0.621	0.1 ± 0.02
Mobile phase volume [75, 100, 125 mL]	1.522	0.39 ± 0.02	0.771	0.82 ± 0.02	0.496	0.1 ± 0.02
Duration of saturation [15, 20, 30 min]	0.381	0.39 ± 0.01	1.148	0.82 ± 0.02	0.608	0.1 ± 0.01
Time from chromatography to scan [20, 30, 40 min]	1.462	0.39 ± 0.01	0.694	0.82 ± 0.01	0.833	0.1 ± 0.01

Table 4 Parameters of system suitability of the proposed TLC-densitometric method for the determination of TL, HZ and AM

Parameter	AM	TL	HZ	Reference value[23]
Capacity factor (K')	9	1.56	0.22	0–10
Symmetry factor	0.93	0.97	1.02	≈1
Resolution (R_s)	3.52	5.25		$R > 2$
Selectivity (α)	5.76	7.09		$\alpha > 1$

system, ethyl acetate : methanol in different ratios (8 : 2, 2 : 8, 5 : 5 v/v), but no good separation was achieved, wherein two of the three peaks usually overlapped or small differences between R_f values were obtained. Other three-component systems were tried, including ethyl acetate : methanol : ammonium hydroxide (5.5 : 4.5 : 0.5 by volume) and ethyl acetate : acetone : ammonium hydroxide (8 : 2 : 0.2 by volume); the addition of ammonium hydroxide to the system did not help much, wherein incomplete resolution of the three drugs was obtained. Also, the ethyl acetate : methanol : acetone system was tried in different ratios to obtain optimum separation of the three cited drugs. The obtained TLC chromatograms were promising, and after fine adjustment of the ratios, the best developing system was found to be ethyl acetate : methanol : acetone (7.5 : 2.5 : 0.5 by volume), on the basis of minimum tailing and maximum separation of the three drugs with the greatest differences between the R_f values: AM – 0.1 ± 0.01, TL – 0.39 ± 0.01 and HZ – 0.82 ± 0.01 (Fig. 2 and 3).

4.1.2. Scanning wavelength. Various scanning wavelengths were checked in order to obtain good sensitivity for TL, HZ and AM with minimum noise. Based on the UV-absorption spectra of TL, HZ and AM, the wavelength of 296 nm is λ_{max} for TL, but not for HZ and AM, while 270 nm is λ_{max} for HZ, but AM showed very poor absorption. Lower wavelengths were avoided to minimize noise and solvent UV-cutoff. The wavelength of 254 nm was found to be optimum, as the three drugs showed good absorbance values at this wavelength, at which sharp and symmetrical peaks with minimum noise were obtained, as shown in Fig. 2 and 3.

4.1.3. Slit dimensions of scanning light beam. The slit dimensions of the scanning light beam have to provide a whole coverage of the band dimensions on the scanned track with no interference from the adjacent ones. Different slit dimensions

Table 5 Determination of telmisartan, hydrochlorothiazide and amlodipine besylate in pharmaceutical dosage form using the proposed method and application of the standard addition technique

Telma-AMH 40®	Found[a] %	Claimed amount (μg per band)	Standard added (μg per band)	Recovery %
TL	100.36 ± 0.622	1	1.0	100.86
			1.5	101.65
			2.5	101.07
		Mean		**101.19**
		RSD%		**0.404**
HZ	99.28 ± 1.05	1	0.5	99.81
			1	98.34
			2	98.17
		Mean		**98.77**
		RSD%		**0.912**
AM	101.47 ± 0.892	2	0.5	98.61
			1	98.07
			1.5	100.87
		Mean		**99.18**
		RSD%		**1.492**

[a] Average of five different determinations.

Table 6 Statistical comparison between the results obtained by the proposed method and the official methods for the determination of telmisartan, hydrochlorothiazide and amlodipine besylate in pure powder form

	TL		HZ		AM	
Parameter	TLC-densitometry	Official method[a]	TLC-densitometry	Official method[b]	TLC-densitometry	Official method[c]
Mean	100.45	99.89	99.96	100.45	99.60	99.93
S.D	0.84	1.03	0.49	0.52	0.68	0.51
RSD	0.836	1.015	0.490	0.517	0.682	0.510
Variance	0.71	1.05	0.24	0.27	0.46	0.26
n	6	6	6	6	6	6
Student *T* test (2.228)[d]	1.034		1.683		0.953	
F (5.05)[d]	1.478		1.125		1.769	

[a] Non aqueous titration; British Pharmacopoeia 2012. [b] Zero order spectrophotometric method at λ_{max} 273 nm; British Pharmacopoeia 2012. [c] HPLC method using C18 column and a mobile phase consisting of acetonitrile : methanol : buffer (15 : 35 : 50 by volume) at a flow rate of 1.0 mL min^{-1} and detection at 237 nm; British Pharmacopoeia 2012. [d] Figures in parentheses are the corresponding tabulated values at $p = 0.05$.

were checked and 3 × 0.45 mm was found to be the one of choice that permits maximum sensitivity.

4.2. Method validation

The proposed TLC-densitometric method was validated according to linearity, range, specificity, accuracy, precision, quantitative limit, detection limit, and robustness according to the ICH guidelines.[23]

4.2.1. Linearity. The linearity of the method was assessed by analysis of different concentrations in the ranges of 1.0–6.0 μg per spot, 0.5–3 μg per spot and 0.5–5.5 μg per spot for TL, HZ and AM, respectively; the linearity of the calibration graphs was validated by high correlation coefficient values and small intercept values (Table 1).

4.2.2. Range. For accurate and precise results; Beer's law and the concentrations of TL, HZ and AM present in the pharmaceutical preparations were considered while establishing the calibration range (Table 1).

4.2.3. Accuracy. The accuracy was tested by applying the suggested method for determination of different blind samples of TL, HZ and AM within the linearity range. The concentrations were obtained from the corresponding regression equations and the results are shown in Table 1, where good percentage recoveries (100.02 ± 0.939, 99.72 ± 0.791 and 100.11 ± 1.186) were obtained for TL, HZ and AM, respectively.

4.2.4. Precision

Repeatability. Three concentrations of TL (2, 4 and 5 μg per band), HZ (1, 2 and 3 and μg per band) and AM (1.5, 2.5, 4.5 μg per band) were analyzed three times intraday using the proposed TLC method; the results are shown in Table 1, where good RSD% values (0.641, 0.834 and 0.774) were obtained for TL, HZ and AM, respectively.

Intermediate precision. The same previously mentioned concentrations were determined on three different days (interday) using the proposed TLC method. The results are shown in Table 1, where good RSD% values (0.893, 0.992 and 0.805) were obtained for TL, HZ and AM, respectively.

4.2.5. Specificity. The specificity of the methods was checked *via* the analysis of different synthetic mixtures of TL, HZ and AM containing different concentrations within the linearity range and the dosage form ratio; the results are shown in Table 2. Good percentage recoveries (100.18 ± 0.791, 99.66 ± 1.011 and 101.22 ± 0.717) were obtained for TL, HZ and AM, respectively.

4.2.6. Determination and quantitation limits (LOD and LOQ). The LOD and LOQ were calculated from the standard deviation (σ) of the response and the slope of the calibration curve (S) according to the following equations: LOD = $3.3(\sigma/S)$ and LOQ = $10(\sigma/S)$. The results presented in Table 1 indicated that the method has acceptable sensitivity for the determination of the studied drugs.

4.2.7. Robustness. In order to evaluate the robustness of the proposed method, the studied ternary mixture was analyzed after altering various parameters in the developed method. The studied parameters were mobile phase composition, mobile phase volume, saturation time and time taken from chromatographic separation to scanning. It is demonstrated that slight intended variations in the previously mentioned parameters have no significant effect on the analysis of the three drugs in their ternary mixture using the proposed TLC method. The low values of RSD% of peak areas along with the nearly identical R_f values indicate good robustness of the proposed method, Table 3.

4.3. System suitability

According to the ICH,[23] system suitability tests are a vital part of many analytical methods, particularly liquid chromatographic methods. They are applied to authenticate that the resolution and reproducibility of the chromatographic system are suitable for the analysis to take place. Parameters, namely capacity factor (k),[24] symmetry factor, selectivity factor (α) and resolution (R_s), were calculated and are shown in Table 4.

4.4. Analysis of pharmaceutical preparations

The proposed methods have been successfully applied to assay TL, HZ and AM in Telma-AMH 40® tablets with good percentage recoveries, which endorses the suitability of the proposed methods for the routine determination of these components in their combined formulation (Table 5).

4.5. Statistical analysis

The results obtained for the analysis of TL, HZ and AM in their pure powder forms using the suggested TLC densitometric method were statistically compared with those results obtained by using the official methods.[16] The calculated *t* and *F* values are less than the tabulated ones, which indicates that there is no significant difference with regard to accuracy and precision (Table 6).

In addition to being a more eco-friendly method compared to the previously reported HPTLC-densitometric method,[15] which used chloroform : methanol : formic acid (85 : 15 : 5 by volume) as a developing system, the suggested method proved to be more linear, with regression parameters that showed higher correlation coefficients and smaller intercepts. In addition, better recoveries were reported for the analysis of TL, HZ and AM in their pure powdered forms and combined tablets. The previously reported method showed higher sensitivity as it was done using HPTLC rather than regular TLC densitometry.

5. Conclusion

Significant greenness can be achieved when GAC principles are applied in our analytical procedure. Advances in sample preparation techniques, instrumentation, chemometric treatment of data, environment-friendly organic modifiers, *etc.*, can drive noteworthy enhancements in both the analytical characteristics and greenness profile of the whole methodology. In the analytical community, we can profit from the green chemistry concepts, since besides the intrinsic advantages of implementing automated, accelerated, simplified and miniaturized systems, there is also a great conservation of solvents, reagents and energy, less risks to the analyst, and less production of waste. More progress is predicted over the coming years, not only towards more eco-friendly analytical methods but also towards greenness assessment.

The proposed TLC densitometric method represents an advantageous method for determination of TL, HZ and AM in their ternary mixture, for being simple (with no tedious extraction steps), accurate, and time- and cost-saving. In addition, the used mobile phase is eco-friendly, wherein the solvents used are considered as greener ones compared to the previously reported ones, without affecting the effectiveness of the method. The TLC-densitometric method was applicable to the assay of TL, HZ and AM in their powder forms, synthetic laboratory prepared mixtures and pharmaceutical formulations without the need for prior separation and with no interference from additives in the pharmaceutical preparation. The advantages of the TLC method are that several samples can be run simultaneously using a small quantity of mobile phase, thus lowering the analysis time and cost per analysis and providing high sensitivity and selectivity. The results obtained indicate that the introduced method can be used as an eco-friendly, simple and accurate method for quality control laboratories.

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