

High Performance Thin Layer Chromatography (HPTLC) Analysis of Anti-hypertensive Combination Therapy in Pharmaceutical Dosage form

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Abstract

A simple, selective, rapid and sensitive high performance thin-layer chromatography (HPTLC)-densitometric method was developed and validated for the simultaneous determination of Amlodipine besylate, Telmisartan and Indapamide in bulk and pharmaceutical dosage form. Chromatographic separation achieved on precoated silica gel 60 F 254 plates using chloroform: toluene: ethyl acetate: methanol: TEA(4:3:1.5:1.5:0.1, v/v) as the mobile phase with densitometric detection at 240 nm. The analytes showed satisfactory separation with R_f values of 0.21 ± 0.0004, 0.53 ± 0.002 and 0.64 ± 0.003 for AMLO, TELMI and INDA, respectively. The method exhibited excellent linearity over the ranges of 250-1250 ng/band for AMLO, 2000-10000 ng/band for TELMI and 125-625 ng/band for INDA. Accuracy studies demonstrated recoveries within 98-102 %, while precision studies showed %RSD values below 2 %. The method remained robust following deliberate variation in mobile phase composition, migration distance and chamber saturation time. Application of the method to the pharmaceutical formulation demonstrated assay values within the acceptable range, with no interference from excipients. Compared with convention HPLC methods, the proposed HPTLC procedure offers rapid analysis, lower solvent consumption, operational simplicity and cost effectiveness making it suitable for routine quality control of AMLO, TELMI and INDA in pharmaceutical formulations.

Keywords

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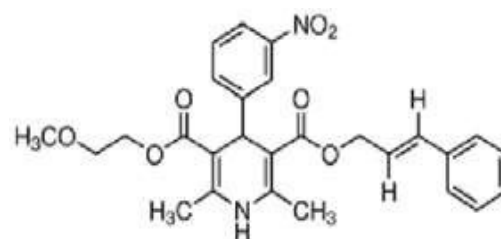
Introduction

Hypertension is one of the leading causes of morbidity and mortality worldwide and is characterized by persistent blood pressure above 140/90 mm Hg. According to global estimates,

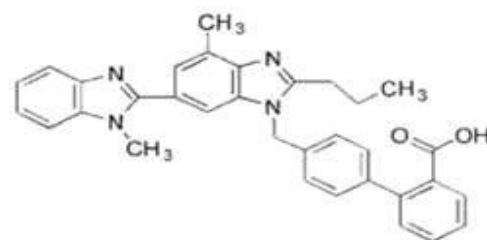
nearly 1.28 billion adults aged 30–79 years suffer from hypertension, with the majority living in low- and middle-income countries. Alarming, about 46% of hypertensive individuals remain unaware of their condition. Many individuals remain unaware of their condition, increasing the risk of severe cardiovascular complications such as stroke, heart failure, and

myocardial infarction. Effective management of hypertension is therefore essential to reduce associated health risks. Commonly prescribed antihypertensive drugs include calcium channel blockers, angiotensin receptor blockers (ARBs), ACE inhibitors, and diuretics. The combination of Amlodipine, Indapamide, and Telmisartan offers a comprehensive and synergistic approach for the effective management of hypertension through multiple mechanisms of action [1-3]. Amlodipine besylate (AMLO) is a widely used antihypertensive drug belonging to the dihydropyridine class of calcium channel blockers. Chemically, it is benzenesulfonic acid; 3-O-ethyl 5-O-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate, with a molecular formula of $C_{26}H_{31}ClN_2O_8S$. Approved by the FDA in 1987, amlodipine is extensively used for the treatment of hypertension and angina. It acts mainly by relaxing peripheral blood vessels, thereby reducing blood pressure. Amlodipine also exhibits antioxidant properties and promotes nitric oxide synthesis, contributing to vasodilation [4] (Figure 1(a)). Telmisartan (TEL), an angiotensin II receptor blocker, is chemically known as 2-[4-[[4-methyl-6-(1-methylbenzimidazol-2-yl)-2-propylbenzimidazol-1-yl]methyl]phenyl] benzoic acid, with a molecular formula of $C_{33}H_{30}N_4O_2$ and acts by selectively blocking angiotensin II AT1 receptors, thereby reducing systemic vascular resistance and aldosterone secretion. Telmisartan is also reported to exhibit partial PPAR γ agonistic activity, which may improve insulin sensitivity and lipid metabolism [5] (Figure 1(b)). Indapamide (IND) is a thiazide-like diuretic commonly prescribed for hypertension and edema associated with congestive heart failure. Chemically, it is 4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoylbenzamide, having a molecular formula of $C_{16}H_{16}ClN_3O_3S$. It effectively lowers systolic blood pressure and reduces oxidative stress. Unlike conventional thiazide diuretics, indapamide has minimal impact on glucose and lipid metabolism, making it suitable for long-term use in hypertensive patients [6] (Figure 1(c)). Fixed-dose combination formulations, such as Widaplik tablets, enhance patient adherence by minimizing pill burden and simplifying the treatment regimen. Such combination therapy also improves therapeutic efficacy and supports long-term management of hypertension. Several analytical methods have been reported in the literature for the quantitative estimation of antihypertensive drugs, either as individual compounds or in combined dosage forms. These methods include spectrophotometric techniques, High-performance liquid chromatography (HPLC), and other chromatographic approaches [7-10]. An

RP-HPLC techniques have been used to determine AMLO, TELMI and INDA simultaneously in combination, according to a survey of the literature [11]. Multianalyte analysis is frequently used in pharmaceutical formulation testing to ascertain the quantity and presence of various excipients and bulk form. The validity and efficacy of pharmaceutical goods depend on this analysis, making it a crucial component of quality control procedures. According to the previous literature review, no trustworthy HPTLC method has been developed for the simultaneous assessment of the three drugs under investigation. HPTLC is a powerful method for evaluating and resolving compounds in pharmaceutical formulations that is often used in pharmaceutical research and industry. Drug resolution, rapid analysis, efficient separation, sample recovery, and suitability for creating and refining analytical methods are only a few benefits of HPTLC. The goal of this study is to develop an HPTLC method for the assay of AMLO, TELMI and INDA that is affordable, sensitive, selective, and dependable. Furthermore, while retaining good analytical performance, HPTLC densitometry offers a cost-effective and efficient substitute for the rather costly and solvent-intensive HPLC methods. The proposed method integrates rigorous statistical evaluation was performed to ensure the accuracy, precision, robustness, and sustainability of the developed analytical procedure in accordance with ICH guideline [12]. Moreover, no tedious and complex extraction and evaporation processes are prerequisites.



(a)



(b)

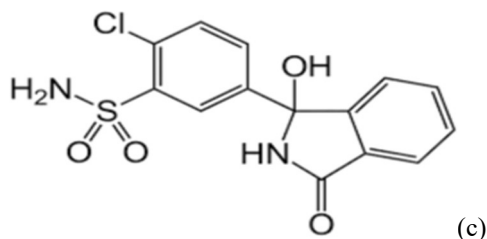


Figure 1. Chemical structure of (a) AMLO (b) TELMI and (c) INDA

2. Material and Methods

2.1 Analytical Instruments

The HPTLC system (Camag, Switzerland) was comprised of the Linomat V semi-automatic sample applicator, TLC scanner-IV (Camag, Muttentz, Switzerland), twin trough development tank (10 × 10 cm), UV cabinet equipped with dual-wavelength UV lamps, VISIONCAT software, and syringe (100 μ L; Hamilton). Electronic analytical balance Shimadzu ATX-220 was employed for all weighing. UV/Visible spectrophotometer UV1800 (Shimadzu) was employed for acquiring the UV spectra. The analysis of experimental data was carried out using VISIONCAT software (version 1.4.8). Pre-coated HPTLC plates (20×10 cm) coated with silica gel 60 F254 on aluminum sheets (Merck) were utilized for the chromatographic analysis. All the plates were pretreated at 110 °C for 5 min before applying samples.

2.2 Reagents and Materials

The APIs of AMLO, TELMI and INDA were collected as gift samples from Ami Life science, Gujrat, India. Since FDCs (Fixed dose combination) is currently unavailable in domestic Indian market, an in-house laboratory scale formulation was developed. The pre-coated TLC plate consisting of silica gel G60F254 (Merck, Germany) was used as stationary phase; Chloroform, toluene, methanol, ethyl acetate and TEA (tri ethyl amine) and various other solvents used in the study are of analytical reagent grade.

2.3 Preparation of standard stock solution : Preparation of Standard stock solution of AMLO (500 μ g/ml), TELMI (4000 μ g/ml) and INDA (250 μ g/ml)

Standard stock solutions of AMLO, TELMI and INDA were prepared by accurately weighing 5 mg, 40 mg, and 2.5 mg of each

drug, respectively, and transferring them into separate 10 mL volumetric flasks. Each drug was dissolved in a small volume of methanol, and the volume was made up to the mark with methanol to obtain final concentrations of 500 μ g/mL, 4000 μ g/mL, and 250 μ g/mL, respectively.

Preparation of combined working standard solution

A combined working standard solution was prepared by transferring 1 mL aliquots of standard stock solutions of AMLO (500 μ g/mL), TELMI (4000 μ g/mL), and INDA (250 μ g/mL) into a 10 mL volumetric flask and mix well.

Selection of wavelength for detection

Aliquots (1 μ L) of standard solutions of AMLO, TELMI and INDA were applied to a TLC plate, developed, and scanned over 200–400 nm using an HPTLC scanner. The overlaid spectra were used to select an appropriate detection wavelength based on absorbance maxima and the ratio of drugs in fixed-dose combinations.

2.4 Chromatographic Separation

An aliquot of 1 μ L from each working standard was applied as 6 mm wide bands onto pre-coated silica gel 60 F254 aluminium plates. The application was performed by maintaining a 10 mm distance from the lower and side edges of the plate, with an inter-band spacing of 10.4 mm. chromatographic development was performed by the linear ascending technique in a twin-trough chamber, pre-saturated with the mobile phase for 20 minutes. The solvent front was allowed to migrate up to a distance of 70 mm under ambient conditions. Post development, the plates were dried and densitometric scanning at 240 nm using a TLC scanner and the data obtained (peak area) was used to generate calibration curves.

2.5 Solution stability

To determine their stability during the validation procedure, the standard and test solutions were checked for an entire day at room temperature. The original area and recovered area were compared. The findings from the study indicated that there was no noteworthy variation in the peak area of 6000 ng/band of TELMI, 750 ng/band for AMLO and 375 ng/band for INDA by % , within the required time span there was no perceptible degradation.

2.6 Method Validation

The developed HPTLC method was validated in accordance with ICH Q2(R2) guideline to ensure its suitability for quantitative analysis of selected analytes.

2.6.1 Specificity

The specificity of the method was evaluated by comparing the spectral profiles of AMLO, TELMI and INDA obtained from standard solutions with those of the corresponding analytes in sample matrix. Spectral overlays at peak start (s), apex (m) and peak end (e) positions revealed a high degree of correlation, indicating the absence of interference from excipients and confirming the peak purity of all three analytes.

2.6.2 Linearity

Linearity was assessed by applying six bands of 0.5-2.5 μ L each, spanning a concentration range of 2000-10000 ng/band of TELMI, 250-1250 ng/band of AMLO and 125-625 ng/band of INDA. Plotting the average peak area ($n = 6$) against the suitable concentration formed the calibration curve.

2.6.3 Sensitivity

The method's sensitivity was assessed by calculating the LOD and LOQ, using the formula given in ICH guideline.

2.6.4 Accuracy

Three points of accuracy-50 %, 100% and 150% of the drug concentration- were estimated using the convention addition method. Pre-analyzed samples were spiked with known concentration of standards, and the consequential samples were then inspected. The recorded peak area was used to compute percent recovery.

2.6.5 Precision

Repeatability, intraday and interday studies were used to determine precision. Applying (6000 ng/band for TELMI, 750 ng/band for AMLO and 375 ng/band for INDA) concentration six times ($n=6$) acceptable for the estimation of repeatability. The % RSD of three concentration (Lower, medium and higher concentration) of the calibration curve, verified three times on the same day and over the course three different days was used to assess intra and inter-day precision, respectively.

2.6.6 Assay

Twenty tablets (In-house laboratory scale formulation) were precisely weighed, grounded into a fine powder, and prepared for quantification to investigate the analyte.

An equivalent amount of powder (i.e. 40 mg of TELMI, 5 mg of AMLO and 2.5 mg of INDA) was weighed and transferred into 100 ml volumetric flask; dissolve and diluted with methanol up to the mark, solution was sonicated and subsequently filtered through Whatman filter paper (N0.42) to exclude any insoluble excipients. An aliquot of 1 μ L of this solution was banded onto a TLC plate; Analyzed using the proposed method and the assay results were calculated accordingly.

2.6.7 Robustness

Robustness studied by observing the effect of small, intentional changes like slight variation in mobile phase composition, migration distance and chamber saturation time. The influence of these changes on the analytical performance was assessed using % RSD values, which indicated that the method remained consistent and reliable under varied conditions.

3. Results and Discussion

Planer HPTLC has become a very reliable and sophisticated planer chromatographic technology that can be applied to both qualitative and quantitative drug analysis as well as regular quality control. The precision and repeatability of the produced chromatograms are greatly enhanced by precise band application employing automated sample applicators, regulated development conditions, and computerized densitometric assessment. When compared to traditional TLC, HPTLC offers superior peak symmetry, increased sensitivity, and high-resolution separation. By measuring the optical density of compact, well-resolved bands directly on HPTLC plates, HPTLC-densitometry is an effective and reliable method for routine analysis that enables precise drug combination identification. Developing a selective, reliable, and sensitive HPTLC approach for AMLO, TELMI and INDA in pharmaceutical formulations and bulk drugs was the goal of the current study. In order to obtain high accuracy, improved resolution, and superior analytical performance appropriate for regular quality control, the approach employs consistent chromatographic conditions.

3.1 Method development and validation

To obtain good chromatographic separation of the chosen analytes, a number of solvent systems with different compositions and

polarities were explored. The mobile phase consisting of chloroform: toluene: ethyl acetate: methanol: TEA (4:3:1.5:1.5:0.1, v/v/v/v/v) produced sharp, symmetrical, and well-resolved peaks with acceptable R_f values (R_f 0.53 ± 0.002 for TELMI, 0.21 ± 0.004 for AMLO and 0.64 ± 0.003 for INDA) (Figure 2), making it the most effective separation among the studied combinations. Triethylamine reduced peak tailing and enhanced peak form, and the solvent system's balanced polarity guaranteed sufficient component migration and resolution. The improved mobile phase showed acceptable selectivity and repeatability, which qualified it for conventional chromatographic analysis and further technique validation. Several wavelengths (200–350 nm) were investigated for densitometric analysis; 240 nm was chosen because it allowed for the simultaneous detection of all analytes with increased sensitivity and low noise (Figure 3). A size of 5×0.45 mm was found to give reliable peak measurement without interference from nearby bands when slit dimensions were optimized. Furthermore, the chamber saturation time was tuned, and it was discovered that 20 minutes produced compact, well-resolved bands, whereas shorter intervals produced poor separation and longer times showed no discernible improvement.

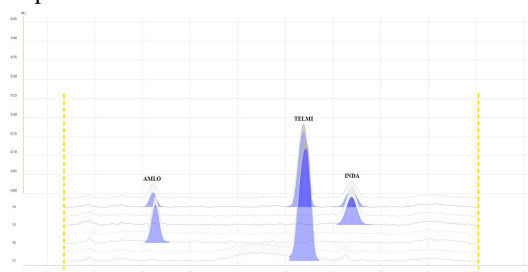


Figure 2. 3D Chromatogram of for AMLO (500 ng/band) , TELMI (4000 ng/band) and INDA(250 ng/band) in mixture and individual Solution.

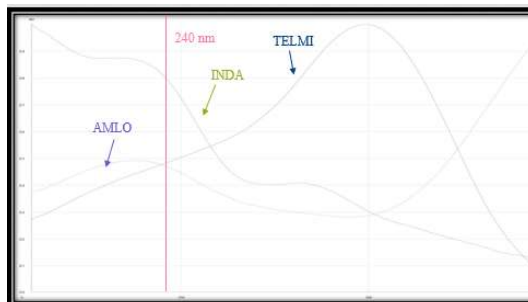


Figure 3. Overlaid in-situ UV spectra of AMLO, TELMI and INDA.

3.2 Validation of the HPTLC Method

The analytical method was verified based on the recommendations outlined in the ICH Q2(R2) guideline for analytical procedure validation [10].

3.2.1 Specificity

The method showed good specificity, as excipients present in the tablet formulation did not interfere with the separation of TELMI, AMLO and INDA (Figure 4). Peak purity was confirmed by comparing spectra at the start, apex, and end of each band, which were found to be consistent for all analytes. Peak purity spectra analysis in chromatograms provide important information about a peak's homogeneity, indicating whether it represents a mixture or a single molecule. This test helps ensure the correctness of analytical results, particularly in quantitative studies, and can detect the presence of impurities or co-eluting substances.

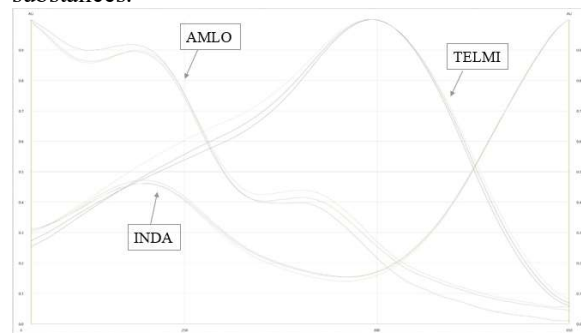


Figure 4: Spectra comparison of standard and sample of drug AMLO, TELMI and INDA.

3.2.2 Linearity and Range

The regression equations and correlation coefficients are shown in table 1, and the overlaid 3 D Chromatogram of drugs across the linearity range are shown in figure 5. With high correlation coefficients, the data confirmed the excellent linearity of the method, demonstrating its reliability for quantitative estimation.

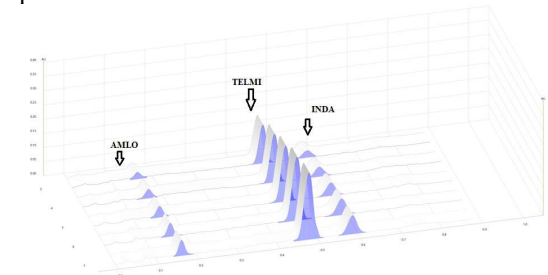


Figure 5: Representative 3D overlain chromatogram for linearity assessment of AMLO, TELMI and INDA.

Table 1: Analytical validation characteristics of the developed HPTLC method for simultaneous determination of AMLO, TELMI and INDA.

Parameters	HPTLC method		
	AMLO	TELMI	INDA
Linearity Range (ng/band)	250-1250	2000-1000 0	125-625
Slope	0.7551	0.592 1	0.266 4
Intercept	75.48	777.6	14.70
Coefficient of determination (R ²)	0.999	0.999	0.998
Accuracy (Mean $\pm$ SD)	99.80 $\pm$ 1.60	100.0 $\pm$ 3 $\pm$ 1.51	100.1 $\pm$ 5 $\pm$ 0.86
Precision Repeatability (% RSD) 0.76 0.04 1.22			
Intraday precision (%	0.32-	0.029	1.06-

RSD)	0.60	-0.16	1.66
Interday precision (% RSD)	0.30-1.04	0.09-0.13	0.83-1.88
LOD(ng/band)	10.72	12.97	15.04
LOQ (ng/band)	32.48	39.31	45.59

3.2.3 LOD & LOQ

The low LOD and LOQ values reflect the good sensitivity of the method, as presented in Table 1.

3.2.4 Accuracy

Accuracy was estimated by calculating systemic errors using the standard addition level at three stages. The recovered amount was calculated using the regression equation, ranged from 98-102 % for all analytes (Table 2), demonstrated that developed technique is highly accurate.

Table 2 (a): Pre-analyzed concentration of AMLO (500 ng/band), TELMI (4000 ng/band) and INDA (250 ng/band)

Dru g	Conc . (ng/b and)	Mean of Area(n =3) $\pm$ SD	Pre-analy zed conc. (ng/b and)	% Me an $\pm$ SD	% R S D
AM LO	500	467.33 $\pm$ 2.08	504.32	100.86 $\pm$ 0.20	0.44
TE LM	4000	3186.0 $\pm$ 5.56	3998.0	99.96	0.17

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I				±	
				1.0	
IND	250	82.66±	252.4	100	1.
A		1.52	0	.96	84
				±	
				0.8	
				7	

Table 2 (b): Recovery studies of AMLO, TELMI and IND

D	L	To	To	To	Spi	Me	
R	e	tal	tal	tal	ked	an	%
U	v	Co	co	co	con	%	R
G	el	nc	nc.	nc.	cent	Rec	S
	%	e.	(n	fo	rati	ove	D
		Sp	g/b	un	on	ry	
		ike	an	d	reco		
		d	d)	(n	vere		
		(n		g/b	d		
		g/b		an	(ng/		
		an		d)	ban		
		d)			d)		
A	5	25	75	75	253.	101	0.
M	0	0	0	8.1	86	.50	5
L				8		±0.	2
O						53	
	1	50	10	10	497.	99.	0.
	0	0	00	02.	98	60	6
	0			30		±	6
						0.6	

						6	
	1	75	12	12	736.	98.	0.
	5	0	50	41.	24	30±	1
	0			56		0.1	7
						7	
T	5	20	60	60	203	100	1.
E	0	00	00	32.	4.58	.08	1
L				58		±1.	1
M						11	
I	1	40	80	79	394	99.	0.
	0	00	00	46.	8.44	42±	8
	0			44		0.8	5
						5	
	1	60	10	10	298.	99.	1.
	5	00	00	02	33	23±	0
	0		0	6.6		1.0	8
				0		8	
I	5	12	37	12	375.	98.	1.
N	0	5	5	04.	24	27±	7
D				43		1.7	6
A						3	
	1	25	50	16	504.	101	1.
	0	0	0	07.	92	.01	5
	0			52		±1.	8
						60	
	1	37	62	20	628.	100	1.
	5	5	5	02.	00	.16	9

	0			96		±2.	9
						00	

3.2.5 Precision

For repeatability, The %RSD values obtained were 0.76 for AMLO, 0.04 for TELMI, and 1.22 for INDA, indicating good precision of the method. Intraday and interday % RSD values (Table 1) were below 2 %, demonstrated good precision, in accordance with ICH guidelines.

3.2.6 Robustness

The findings summarized in Table 3 demonstrate the robustness of the method, showing no significant impact from small changes in mobile phase parameters, migration distance, or saturation time.

Table 3: Robustness study of AMLO, TELMI and INDA

Change s in Parame ters	AMLO		TELM		INDA	
	Rf	Ar ea	Rf	Ar ea	Rf	Ar ea
Mobile	1.	0.3	1.	1.1	1.	1.5
Phase	59	3	63	1	44	8
Compos						
ition (						
0.5 ml						
methano						
l ± 1 %)						
Change	1.	0.1	1.	1.2	1.	1.8
in	52	2	15	6	56	6
migratio						
n						
Distance						
(70 ±5						
mm)						

Change	1.	0.1	1.	1.2	1.	1.8
in	82	3	20	0	46	2
saturation						
n Time (						
20 ± 5						
minute)						

3.2.7 Assay

Table 4 concise the assay's results, which showed that it satisfied 98% and 102% of the agreement criteria. Excipients did not reveal any extra peaks, and the combination's retention duration was found to be identical to the standard, as illustrated in Figure 4.

Table 5: Assay of AMLO, TELMI and INDA in Pharmaceutical Dosage Form (n=6)

Dr ug	Ta bl et co nt en t (m g)	Co nc en (ng/ ban d)	Me an con c.fo und (ng/ ban d)	% puri ty ±SD	% R S D	Mea n table t Cont ent foun d ± SD
A M L O	5	500	505. 77	101. 15±0 .71	0.70	5.05 ±0.0 3
TE L MI	40	4000	406 9.68	101. 74±0 .06	0.06	40.6 9±0. 02
IN D A	2.5	250	250. 27	100. 27 ± 1.25	1.24	2.50 ± 0.03

3.3 The Method's Comparisons with Previously Reported HPLC Method

A comparison between the analytical performance of the developed HPTLC method and previously reported HPLC methods is presented in Table 6. The comparison highlights key parameters to evaluate the suitability and effectiveness of the proposed method relative to existing approaches. The HPTLC method exhibited

good linearity within the studied range, along with acceptable sensitivity as indicated by its LOD and LOQ values. Although the HPLC method showed comparatively higher sensitivity, the HPTLC method provided superior precision, as reflected by lower %RSD values. A significant advantage of the proposed method is its rapid analysis time (less than 2 minutes) compared to the longer run time required for HPLC, making it highly suitable for high-throughput analysis. Additionally, the simpler mobile phase composition and lower solvent consumption make the HPTLC method more economical and environmentally friendly. Overall, the developed HPTLC method can be considered a reliable, precise, and cost-effective alternative for routine quality control analysis, whereas the HPLC method remains preferable when higher sensitivity is required.

Table 6: Comparison between the proposed HPTLC method and the reported HPLC method for the determination of AMLO, TELMI and INDA [11]

Parameters	Proposed HPTLC method	Reported HPLC method
Stationary Phase	Pre coated silica gel G60- F254 TLC Aluminum sheet (E. Merck, Germany) (20×10 cm, thickness layer 0.2 mm)	HIBAR ODS C18, 250 mm*4.6 mm
Mobile Phase	Chloroform: toluene: ethyl acetate: methanol: TEA	Acetonitrile: 0.05 M Potassium

	(4.0 :3.0:1.5:1.5:0.1V/V/V)	dihydrogen phosphate (50:50 v/v), pH 3.0
Detection wavelength	240 nm	238 nm
Linearity	AMLO: 250-1250 ng/band TELMI:2000-10000 ng/band INDA:125-625ng/band	AMLO: 1.25 – 7.5 µg/mL TELMI: 10 – 60 µg/mL INDA: 0.625 – 3.75 µg/mL
Run time/per Sample	Less than 2 min (Elution time+ saturation time/number of samples per plate)	20 min Without including the actual time required for

		normalizing the baseline
LOD	AMLO: 10.72 ng/band TELMi:12.97 ng/band INDA: 15.04 ng/band	AMLO: 0.057 µg / ml TELMi: 0.249 µg / ml INDA:0.0 55 µg / ml
LOQ	AMLO: 32.48 ng/band TELMi:39.31 ng/band INDA: 45.59 ng/band	AMLO: 0.174 µg / ml TELMi:0. 754 µg / ml INDA: 0.167 µg / ml
Repeatability (%RSD)	AMLO: 0.76 TELMi:0.04 INDA: 1.22	AMLO: 1.07 TELMi:0. 52 INDA: 1.40

4. Conclusion

A simple, sensitive, precise, and selective HPTLC–densitometric method was successfully developed for the simultaneous

quantification of AMLO, TELMI and INDA in both bulk drug and pharmaceutical dosage forms. The method demonstrated reliable performance, with statistical validation confirming its suitability for routine analysis without interference from excipients. In addition to its application in formulation analysis, the method shows potential for extension to degradation studies and analysis in biological matrices. Compared to conventional HPLC techniques, the proposed HPTLC method offers advantages such as lower cost, simplicity, rapid analysis, and operational flexibility. Moreover, the method eliminates the need for any prior separation step, making it particularly suitable for routine and repetitive analysis of the studied drugs.

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Conflict of Interest

The author/editor has no conflicts of interest, financial or otherwise, to declare.

Statement of Contribution of Researchers

Concept – D.P, G.P., PKP.; Design – D.P., Y.P.; Supervision – D.P.,G.P., D.B.M; Data Collection and/or Processing – Y.P., S.P., D.S., Analysis and/or Interpretation – M.P., Y.P., D.J., S.P.; Literature Search – Y.P; Writing – Y.P.M.P., D.J.; Critical Reviews – D.P., G.P., D.B.M.

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