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ORIGINAL RESEARCH PAPER

Simultaneous determination of amlodipine besylate and azilsartan mixture in human plasma utilizing high-performance thin-layer chromatography with ultraviolet detection

Ahmed A. Khorshed[1,2] · Fatma M. Abdelnaeem[1] · Sayed M. Derayea[3] · Mohamed Oraby[1] · Dalia M. Nagy[3]

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Abstract
A novel, efficient, and sensitive high-performance thin-layer chromatography (HPTLC) method has been developed and validated for the concurrent measurement of amlodipine besylate (AML besylate) and azilsartan (AZL) in human plasma spiked with the mixture. Reflectance/absorbance densitometry was conducted using toluene−ethyl acetate−methanol−acetone−acetic acid (6:1.5:1:0.5:1, *V/V*) as the mobile phase, and separation was achieved on a precoated silica gel HPTLC plate. This chromatographic system yielded compact bands with excellent resolution at a retardation factor (R_F) of 0.22 ± 0.002 for AML besylate and 0.73 ± 0.001 for AZL. Quantification of AML besylate and AZL was performed at 244 nm within the ranges of 60−600 ng per band and 90−900 ng per band, respectively. Calibration plots exhibited strong linearity, with correlation coefficients of 0.9976 for AML besylate and 0.9974 for AZL. Following the International Council for Harmonisation (ICH) guidelines, the developed method was validated. The lowest detectable values for AML besylate and AZL were 13.79 ng per band and 18.62 ng per band, respectively. The recommended HPTLC methodology for the simultaneous determination of AML besylate and AZL is demonstrated to be sensitive, selective, accurate, and precise. This technique can effectively be applied to the simultaneous detection and quantification of AML besylate and AZL in synthetic mixtures and human plasma samples. The enhancing effect of ammonia on the absorption intensity and the bathochromic effect on the wavelength of absorbtion were investigated by molecular modeling and it is suggested that ammonia causes acrylamide to change into acrylamic acid with more conjugated double bonds that rationale the increase in the absorption intensity and the bathochromic shift in the wavelength of the absorption.

Keywords High-performance thin-layer chromatography (HPTLC) · Reflectance/absorbance · Amlodipine besylate · Azilsartan · Plasma

1 Introduction

Amlodipine besylate (AML besylate) is an antihypertensive drug that belongs to the dihydropyridine calcium channel blockers class and is administered orally [1]. Its chemical name is 3-ethyl-5-methyl-(4*RS*)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulfonate [2]. AML besylate inhibits the initial calcium influx by blocking the voltage-dependent L-type calcium channels [3]. Boasting a lengthier half-life (30–50 h) in comparison to nifedipine and other drugs in the dihydropyridine class, AML besylate permits once-daily dosing and has been widely utilized for two decades [3]. The US Food and Drug Administration (FDA) first approved AML besylate in 1987 [1]. AML besylate is distinguished as an optimal first-line choice among various antihypertensive drugs for the treatment of hypertension. It can be taken either alone or in combination with other antihypertensive medications [3]. Prescribed to alleviate stable, long-term angina symptoms and treat vasospastic angina, whether proven or suspected [4]. It exhibits immediate vasodilation and prolonged antihypertrophic activity,

✉ Ahmed A. Khorshed
ahmed_khorshed@pharm.sohag.edu.eg

[1] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Sohag University, Sohâg 82524, Egypt

[2] Department of Biomedical Engineering, University of Alberta, Edmonton, AB T6G 1H9, Canada

[3] Analytical Chemistry Department, Faculty of Pharmacy, Minia University, Minia 61519, Egypt

contributing to its beneficial effects on the peripheral and coronary vascular bed [5].

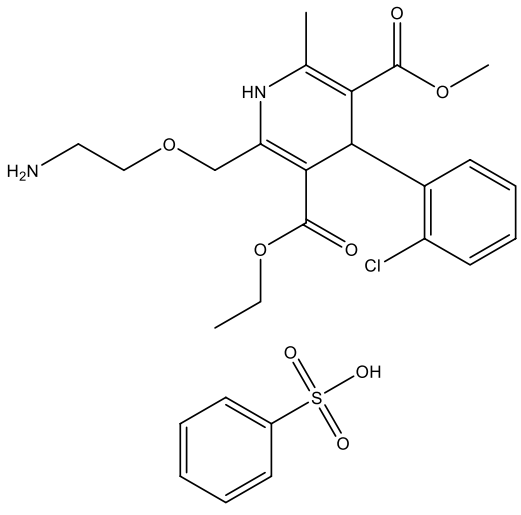
The FDA granted approval to a new angiotensin receptor blocker called azilsartan (AZL) on 25 February 2011 for treating hypertension [6]. It is intended for use in adults aged 18 and older [7]. The chemical name is 2-ethoxy-1-{[2′-(5-oxo-4,5-dihyro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl}-1H-benzimidazole-7-carboxylic acid [8]. AZL is a prodrug that rapidly hydrolyzes to the active component AZL in the gastrointestinal tract upon oral administration before systemic absorption [9]. By specifically inhibiting angiotensin II from binding to the angiotensin type 1 (AT1) receptor and counteracting the pressor response activity of angiotensin II, it exerts its antihypertensive effects [9]. Cytochrome P450 (CYP) 2C9 is the primary enzyme responsible for AZL metabolism. AZL undergoes two main metabolites, M-I and M-II, through decarboxylation and O-dealkylation, respectively. The pharmacologic activity of AZL remains unaffected by these metabolites due to their poor affinity for the AT1 receptor [7].

The coadministration of AML besylate 5 mg daily plus azilsartan (Fig. 1), either 40 mg or 80 mg, resulted in a significant decrease in systolic blood pressure compared with AML besylate alone [6]. Effective results were obtained when AZL was taken in combination with AML besylate or with chlorthalidone [10]. Therefore, there is a considerable need to develop an analytical method for their simultaneous determination.

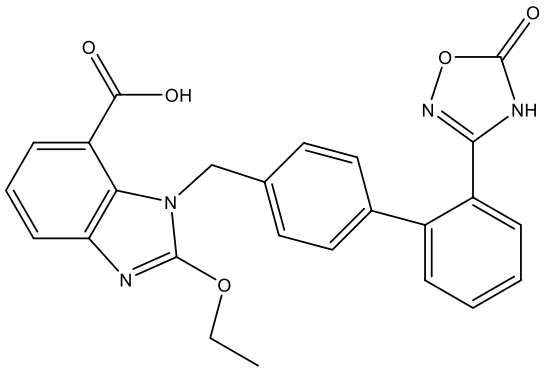
Various methods, including high-performance liquid chromatography (HPLC) [11, 12], high-performance thin-layer chromatography (HPTLC) [13, 14], spectrophotometry [15, 16], spectrofluorimetry [17, 18], and voltammetry [19–24], have been reported for determining AML besylate. Similarly, for AZL, reported approaches encompass spectrophotometry [8, 25], spectrofluorimetry [26, 27], HPLC [28, 29], HPTLC [30, 31], voltammetry [32]. Only one spectrophotometric method has been published for the simultaneous estimation of AML besylate and AZL [33]. One notable disadvantage of spectrophotometry in drug analysis within biological fluids is its susceptibility to interference from complex matrices, leading to diminished selectivity and potential inaccuracies in quantification due to the presence of overlapping absorbance signals from various components in the biological sample.

Chromatographic techniques excel in drug detection in plasma due to its exceptional selectivity, showcasing a remarkable capability to effectively separate drugs from interfering components within the complex plasma matrix [34]. This distinctive feature ensures accurate and reliable results by minimizing interference and enhancing the precision of drug analysis.

Given the manifold advantages of HPTLC, it is currently a widely utilized technology for quantitative analysis [34–38]. HPTLC stands out due to its cost effectiveness, rapid analysis, low maintenance costs, and enhanced accuracy and precision [39–41]. Notably, there is no need to treat the sample separately, as it can be processed alongside the standard on the same plate. With each test starting with a new stationary phase, the risk of contamination or interference from previously examined samples is eliminated. Unlike HPLC, where strongly held solutes may obstruct the column, the visual identification of HPTLC of every component on the plate mitigates such concerns [42]. The HPTLC



Amlodipine besylate



Azilsartan

Fig. 1 The chemical structures of the studied drugs

technique enables the simultaneous analysis and quantification of numerous compounds [43].

However, as of now, no HPTLC method has been published for the concurrent analysis of AML besylate and AZL. The objective of this study was to develop a rapid, straightforward, cost-effective, efficient, sensitive, and selectively reliable HPTLC method utilizing reflectance/absorbance mode for the simultaneous determination of AML besylate and AZL. This approach offers the advantage of cost savings, reduced effort, time efficiency, and minimized solvent usage. The recommended analytical process underwent validation in accordance with the International Council for Harmonisation (ICH) requirements.

2 Experimental

2.1 Chromatographic analysis setup

The chromatographic analysis utilized a CAMAG HPTLC system (CAMAG, Muttenz, Switzerland) with Scanner 3 operated using winCATS version 1.4.4.6337 software HPTLC. A high-pressure deuterium and halogen tungsten lamp served as the radiation source in the reflectance/absorbance mode. The scanner featured a slit with measurements of 3 mm × 0.45 mm, and the scanning speed was set at 20 mm/s. Sample application on the plate employed a Hamilton syringe (100 µL, Bonaduz, Switzerland) and a Linomat 5 semi-autosampler under a gentle nitrogen stream with 100 µL predosage volume, and the dosage speed was 100 nL, corresponding to four steps per band. The plate underwent development in the ascending mode within a twin-trough chamber measuring 27.0 cm × 26.5 cm × 7.0 cm.

2.2 Chemicals, standards, and samples

AML besylate was obtained from Global Napi Pharmaceutical Co. (6 October, Egypt), and AZL was obtained from Rameda Pharmaceutical Company (6 October, Egypt). Both AML besylate and AZL exhibited a purity of not less than 98.00%, regularly tested by HPTLC analysis. Solvents, including toluene, ethyl acetate, methanol, acetone, and acetic acid, were procured from El-Nasr Pharmaceutical Chemicals in Abo-Zaabal (Cairo, Egypt).

2.3 Preparation of standard solution

Separate stock solutions of 1.0 mg/mL for AML besylate and AZL were prepared by dissolving 10.0 mg of each drug in 7 mL methanol, sonicating for 10 min, and completing the volume to 10 mL with the same solvent. Standard working solutions were then obtained through mixing certain concentration of both drugs and dilution with methanol to achieve final concentrations of 20, 40, 80, 120, 160, and 200 µg/mL for AML besylate and 30, 50, 100, 140, 200, and 300 µg/mL for AZL.

2.4 Preparation of plasma samples

Human plasma samples were prepared by combining 150 µL of freshly prepared human plasma, 150 µL of AML besylate standard solution, and 150 µL of AZL standard solution. Following vortexing for 2 min, 300 µL of acetonitrile were added, and after 5 min of vortexing, 750 µL of methanol were added. The mixture underwent centrifugation for 15 min at −4 °C at 14,000 rpm. The supernatant was treated according to the outlined chromatographic conditions.

2.5 Chromatographic conditions

Aluminum HPTLC plates (Merck, Darmstadt, Germany; catalog no. 1055480001) precoated with silica gel 60 F_{254}, with a stationary phase thickness of 200 µm and a particle size of 5 µm, were employed. The plates were cut into pieces measuring 20 cm × 6 cm, and a methanol prewash was applied before chromatographic development. The optimal mobile phase composition determined after several trials was toluene−ethyl acetate−methanol−acetone−acetic acid (6:1.5:1:0.5:1, *V/V*). The mobile phase volume was 10 mL, and the chamber was saturated with mobile phase vapor at room temperature for 20 min. After development and drying the plate for 10 min in air, the plate was transferred to the HPTLC scanner for the ultraviolet (UV) measurements, which were conducted at 244 nm (Fig. 2) utilizing reflectance/absorbance mode.

3 Results and discussion

The concurrent use of AML besylate and AZL was observed to produce substantial antihypertensive effects [44]. This study presents the first HPTLC method for the simultaneous quantification of AML besylate and AZL, providing a

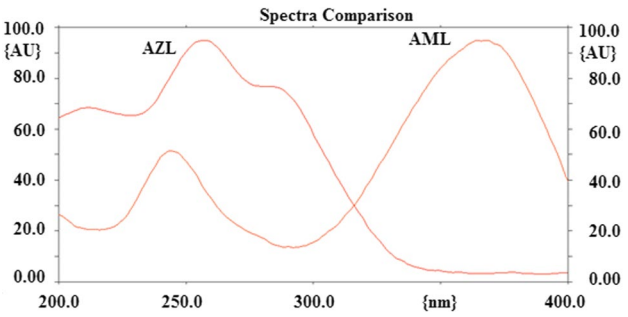


Fig. 2 Absorption spectra of amlodipine besylate and azilsartan

straightforward, rapid, cost-effective, and accurate approach. The method exhibited high sensitivity and selectivity, allowing for the determination of both drugs in spiked human plasma after coadministration.

Since all previously published HPTLC methods were applied either to individual medications or in combination with other different drugs, there is a pressing need to establish an innovative quantitative methodology for the concurrent quantification of the drugs under study. There is no requirement for extraction, and the AML besylate and AZL measurement was accomplished in a single run, saving time, resources, and employment. Because of its highly sensitive and selective performance, the suggested method was able to determine both drugs in spiked human plasma, allowing determination of the blood levels of the studied drugs after coadministration.

After multiple attempts with varying ratios of different organic solvents, the optimal mobile phase was determined to be a mixture of toluene–ethyl acetate–methanol–acetone–acetic acid (6:1.5:1:0.5:1, *V/V*), and the optimal detection wavelength was 244 nm in the reflectance/absorbance mode. This resulted in highly resolved and compact bands with retardation factor (R_F) 0.22 ± 0.002 for AML besylate and 0.73 ± 0.001 for AZL (Fig. 3).

3.1 Method development

Several mobile phase compositions were explored for the HPTLC−densitometry analysis to achieve optimal separation of the AML besylate–AZL binary mixture (Table 1). The best mobile phase composition was toluene−ethyl acetate−methanol−acetone−acetic acid (6:1.5:1:0.5:1, *V/V*) with R_F 0.22 ± 0.002 for AML besylate and 0.73 ± 0.001 for AZL (Fig. 3). This resulted in highly resolved and compact bands, with 244 nm as the optimum wavelength for the simultaneous detection,with this offering the highest sensitivity for both drugs.

3.2 Method validation

The recommended analytical process underwent validation in accordance with the International Council for Harmonisation (ICH) requirements.

3.2.1 Linearity

To establish calibration curves, the peak area responses were plotted against drug concentrations. Linearity was demonstrated in the concentration ranges of 60–600 ng per band for AML besylate and 90–900 ng per band for AZL (Fig. 4). Statistical parameters for the linear plots of both drugs are outlined in Table 2, revealing correlation coefficients (r) of 0.9976 for AML besylate and 0.9974 for AZL, along with determination coefficients (r^2) of 0.9952 and 0.9948, respectively. These calculated coefficients signify a strong correlation between the investigated concentrations and the corresponding peak areas.

3.2.2 Limit of detection and limit of quantification

Sensitivity assessments were conducted by calculating limit of detection (LOD) and limit of quantification (LOQ) based on the standard deviation of the intercept and the slope of the calibration curve. LOD was expressed as 3.3 σ/S, while LOQ was expressed as 10 σ/S. The calculated values for LOD and LOQ were 13.79 and 41.78 ng per band for AML besylate and 18.62 and 56.42 ng per band for AZL (Table 2). These smaller LOD and LOQ values enhance the method's capability for the sensitive determination of AML besylate and AZL in spiked human plasma.

3.2.3 Accuracy

The accuracy of the proposed HPTLC method was assessed by determining three different concentration levels for each drug: 120, 360, and 480 ng per band for AML

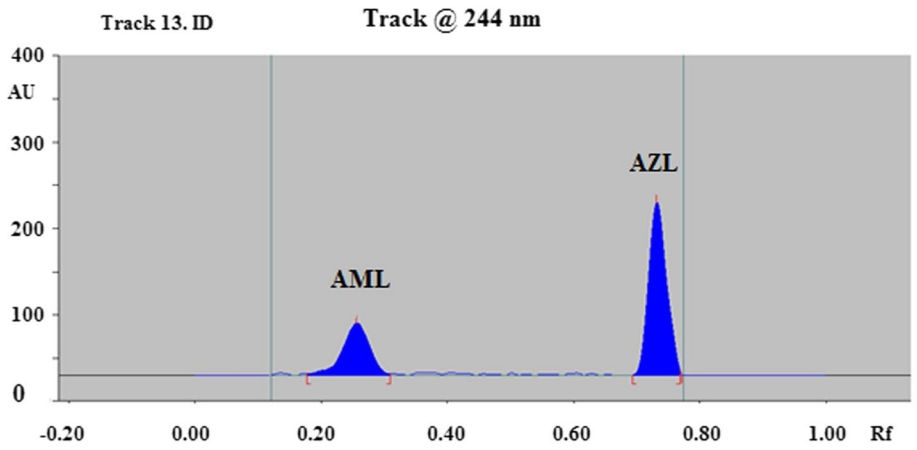


Fig. 3 HPTLC densitogram of the mixture containing 480 ng per band of amlodipine besylate and 600 ng per band of azilsartan

Table 1 Effect of different mobile phase solvent systems on the retardation factor for the separation of azilsartan–amlodipine besylate mixture

Mobile phase (*V/V*)	Amlodipine besylate	Azilsartan
Dichloromethane–methanol–acetic acid 4:5.5:0.5	0.72	At the solvent front
Dichloromethane–methanol–acetic acid 7:2.7:0.3	0.71	At the solvent front
Toluene–ethyl acetate–methanol–acetic acid 4:4:1.8:0.2	0.09	0.72
Toluene–ethyl acetate–methanol–acetic acid 3.5:4:2.3:0.2	0.14	0.81
Toluene–ethyl acetate–acetone–acetic acid 3.5:4:2.3:0.2	At baseline	0.70
Toluene–ethyl acetate–acetone–acetic acid 3:3:3.8:0.2	At baseline	0.75
Toluene–ethyl acetate–methanol–acetone–acetic acid 3:4:2.3:0.5:0.2	0.15	0.81
Toluene–ethyl acetate–methanol–acetone–acetic acid 3:4:2:0.5:0.5	0.19	0.89
Toluene–ethyl acetate–methanol–acetone–acetic acid 4:3:1:0.5:1.5	0.30	0.85
Toluene–methanol–acetic acid 6:2:2	No resolution between two drugs	
Toluene–ethyl acetate–methanol–acetonitrile–acetic acid 6:1.5:1:0.5:1	0.26 with tailing	0.73
Toluene–ethyl acetate–methanol–acetonitrile–acetic acid 6:1.5:1:1:0.5	0.21 with tailing	0.71
Toluene–ethyl acetate–methanol–acetonitrile–acetic acid 4.5:1.5:1:1:2	0.46	0.85
Toluene–ethyl acetate–acetonitrile–acetic acid 4.5:2.5:1:2	0.17 with tailing	0.81
Toluene–ethyl acetate–methanol–acetone–acetic acid 6:1.5:1:0.5:1	0.22	0.73

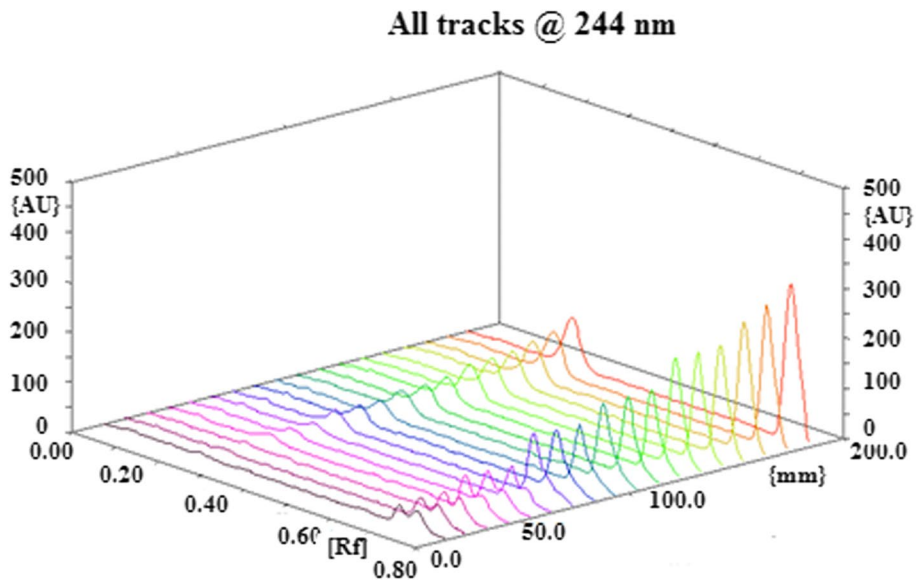


Fig. 4 Three-dimensional graph showing recorded intensities against R_F values for 60–600 ng per band for amlodipine besylate and 90–900 ng per band for azilsartan measured by reflectance/absorbance mode at 244 nm

Table 2 Statistical data of some analytical parameters of the proposed HPTLC−UV method for the simultaneous determination of amlodipine besylate and azilsartan

Parameter	Amlodipine besylate	Azilsartan
Linear range (ng per band)	60−600	90−900
Slope ± SD[a]	2.51 ± 0.08	5.97 ± 0.21
Intercept ± SD[a]	23.04 ± 10.48	45.98 ± 33.70
Correlation coefficient, r	0.9976	0.9974
Determination coefficient, r^2	0.9952	0.9948
R_F	0.22 ± 0.002	0.73 ± 0.001
LOD (ng per band)	13.79	18.62
LOQ (ng per band)	41.78	56.42

[a]Average of three determinations

Table 3 Assessment of the accuracy of the proposed HPTLC−UV method for the simultaneous determination of amlodipine besylate and azilsartan

Drug	Concentration of drug (nanograms per band)	Amount found (nanograms per band)	Percent recovery[a] (%) ± standard deviation
Amlodipine besylate	120	122.69	102.24 ± 0.99
	360	360.94	100.26 ± 1.02
	480	475.68	99.10 ± 0.63
Azilsartan	150	150.59	100.39 ± 1.90
	300	297.49	99.16 ± 2.06
	600	605.36	100.89 ± 1.61

[a]Average of six determinations

besylate and 150, 300, and 600 ng per band for AZL. Six replicate measurements were performed for each concentration level. The method demonstrated high accuracy, evident from the close proximity of the obtained percent recovery values to 100% and the small standard deviation values (Table 3).

3.2.4 Precision

Precision was evaluated for both intraday and interday measurements, expressed as relative standard deviation (RSD). The repeatability of the proposed HPTLC method was studied through six replicates at three different concentration levels for AML besylate (120, 360, and 480 ng per band) and AZL (150, 300, and 600 ng per band), which represents the intraday precision. The assessment of interday precision involved analyzing the same concentration ranges over three consecutive days. The results presented in Table 4 demonstrate that the RSD did not exceed 2.72% for all concentration levels, indicating the commendable precision of the suggested HPTLC method.

Table 4 Evaluation of the precision of the proposed HPTLC−UV method for the simultaneous determination of amlodipine besylate and azilsartan at the intra- and interday levels

Drug	Concentration of drug (ng per band)	Intraday	Interday
		%RSD[a]	*%RSD*[b]
Amlodipine besylate	120	2.44	2.71
	360	1.94	0.91
	480	2.17	0.74
Azilsartan	150	2.28	2.25
	300	1.44	2.72
	600	1.61	0.90

[a]Estimated from six determinations at each concentration level

[b]Estimated from 18 determinations at each concentration level over 3 days

Table 5 Investigation of the robustness of the proposed HPTLC−UV method for the simultaneous determination of amlodipine besylate and azilsartan

Parameter	Amlodipine besylate	Azilsartan
Optimal parameters	100.26 ± 1.02	100.68 ± 0.77
Composition of the mobile phase: toluene−ethyl acetate−methanol−acetone−acetic acid		
(5.8:1.7:1:0.5:1, *V/V*)	101.26 ± 1.46	99.15 ± 2.20
(6:1.5:0.8:0.7:1, *V/V*)	100.86 ± 1.90	101.37 ± 1.08
Scanning wavelength		
242 nm	98.65 ± 2.25	99.36 ± 1.96
246 nm	99.74 ± 1.85	101.24 ± 1.38
Saturation time		
18 min	98.47 ± 1.26	101.40 ± 1.85
22 min	99.42 ± 2.43	100.87 ± 0.68

[a]Average of six determinations

3.2.5 Robustness

To gauge the robustness of the proposed method, we intentionally made minor adjustments to certain parameters, including the mobile phase composition, scanning wavelength, and saturation time. The effects of these alterations on the peak area of the studied drugs were meticulously assessed. This involved calculating the recovery percentage and standard deviation for each parameter, comparing them with the conditions optimized for the study.

The obtained results revealed that even with slight variations in the proposed method variables, the percentage recovery consistently fell within the range of 98.47 ± 1.26 to 101.40 ± 1.85%. This finding underscores

the commendable robustness and reliability of the proposed method in the analysis of the studied drugs. For a detailed breakdown, see Table 5.

3.3 Application to spiked human plasma

The efficiency of the developed HPTLC method in chromatographically isolating both AML besylate and AZL from potential interfering compounds, owing to its robust separation capabilities and enhanced sensitivity, enabled the accurate detection of the targeted compound mixture in complex plasma matrices. Determining the blood levels of the studied drugs after coadministration holds significant value. Due to challenges in collecting blood samples from patients undergoing the investigated drug regimen, we opted to conduct the analysis on human plasma samples that had been mixed with AML besylate and AZL in vitro. The analysis revealed a robust separation of the two drugs on the HPTLC plate with clear identification.

To validate this capability, a meticulous assessment was conducted by determining four concentrations of AML besylate and AZL, spanning their respective linear ranges in spiked human plasma and employing a replicative approach of six measurements for each concentration level. An aliquot of plasma sample without spiking with the targeted drugs was analyzed as a method blank (Fig. 5a). In addition, the plasma samples were spiked with the studied drug mixture at four different concentration levels for each drug as illustrated in Fig. 5b. The subsequent computation of recovery percentages and standard deviations provided quantitative insights into the method's reliability and precision as the

Table 6 Application of the proposed HPTLC–UV method for the simultaneous determination of amlodipine besylate and azilsartan in spiked human plasma

Drug	Concentration of drug (nanograms per band)	Amount found (nanograms per band)	Percent recovery[a] ± standard deviation
Amlodipine besylate	120	114.92	95.77 ± 1.12
	360	373.95	103.87 ± 1.86
	480	467.77	97.45 ± 1.90
	600	590.75	98.46 ± 2.13
Azilsartan	150	144.80	96.53 ± 1.81
	300	291.18	97.06 ± 2.45
	420	401.67	95.63 ± 0.89
	600	613.86	102.31 ± 1.26

[a]Average of six determinations

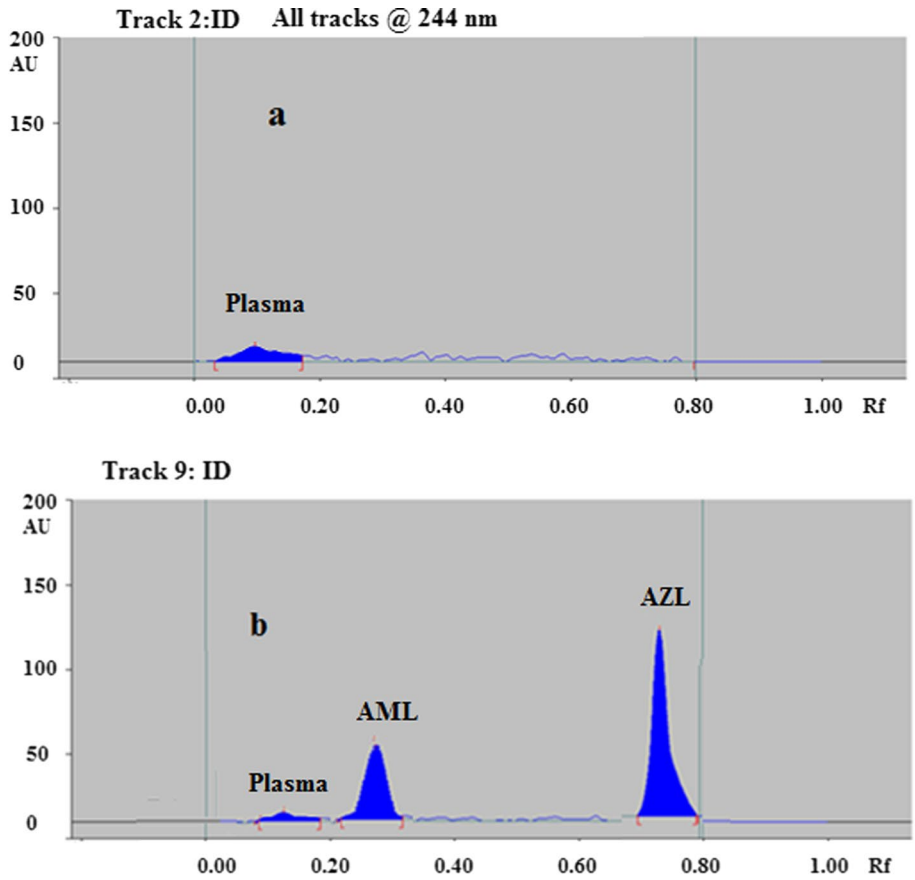


Fig. 5 HPTLC densitogram of plasma sample **a** without spiking with amlodipine besylate and azilsartan and **b** spiked with 360 ng per band of AML besylate and 300 ng per band of azilsartan

recovery percentages ranged from 95.77 to 103.87 for AML besylate and from 95.63 to 102.31 for AZL at four different concentration levels (Table 6). The consistently high percentage of the obtained recoveries supports the suitability of the recommended approach for quantifying the investigated medicines in spiked plasma. The standard deviations obtained ranged from 0.89 to 2.45, signifying a low level of variability influenced by the matrix during the measurement of AML besylate and AZL in human plasma.

4 Conclusions

This study introduces a novel HPTLC method with reflectance/absorbance mode for the simultaneous quantification of the AML besylate−AZL combination. The approach is characterized by its simplicity, speed, accuracy, cost-effectiveness, and applicability without the need for complex extraction procedures. It demonstrates high sensitivity and selectivity in determining both drugs in spiked human plasma after coadministration. In comparison with HPLC methods, the proposed HPTLC method offers a more favorable alternative, avoiding the drawbacks of expense, time consumption, and complex sample preparation.

Declarations

Conflict of interest The authors declare that they do not have a conflict of interest.

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