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Validated Chromatographic Methods for Simultaneous Determination of Amlodipine Besylate and Perindopril Arginine in Binary Mixtures and in Pharmaceutical Dosage Form

Nouruddin W. Ali[1], Nada S. Abdelwahab[1], Marco M. Zaki[1]* and M. Abdelkawy[2]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Beni-Suef University,Alshaheed Shehata Ahmed Hegazy st.62514, Beni-Suef, Egypt
[2]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., 11562 Cairo, Egypt

Abstract

Two accurate, selective and precise chromatographic methods, namely TLC-densitometric method & RP-HPLC method & were developed and validated for the simultaneous determination of Amlodipine besylate and Perindopril Arginine in their binary mixtures.

The developed TLC-densitometric method depends on separation and quantitation of the studied drugs on silica gel 60F_{254} TLC plates. Chloroform:methanol:deionized water:glacial acetic acid:triethylamine (10:7:5:0.3:0.2, by volume) was used as developing system and the separated bands were scanned at 208 nm. Linear relationship was obtained in the range 1-10 µg for both drugs.

The developed RP-HPLC depends on quantitative chromatographic separation of the studied drugs on a C18 column using phosphate Buffer:acetonitrile (60:40, v/v), pH=4.6 as a mobile phase delivered at constant flow rate of 1 mL.min^{-1} with UV detection at 210 nm. Calibration curves for Amlodipine besylate and Perindopril Arginine were constructed over the concentration range of 1-50 µg.mL^{-1} for both drugs.

The proposed methods were successfully applied for determination of the studied drugs in their bulk powder and in pharmaceutical formulations. The proposed methods were statistically compared to each other using student's t test and F-test and no significant difference was found between them.

Keywords: Amlodipine; Perindopril; TLC-densitometry; RP-HPLC

Introduction

Amlodipine besylate (AB) is 3-ethyl 5-methyl 4RS-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzene sulphonate [1], (Figure 1). It is a calcium channel blocker [2], used as an anti-hypertensive and in the treatment of angina, it lowers the blood pressure, relaxes heart muscles and dilates the heart blood vessels to prevent spasm [3]. Perindopril L-arginine (PA) is (2S, 3aS, 7aS)-1-N-[(S)-1-ethoxycarbonyl butyl]-L-alanyl) perhydroindole-2–carboxylate [4] (Figure 2). It is an angiotensin converting enzyme (ACE) inhibitor used for the treatment of hypertension. It may be used alone or in combination with other antihypertensive agents [3].

The literature survey revealed that there are some analytical methods for the determination of Perindopril Erbumine including Potentiometric [1], HPLC [5], LC-MS [6] and CE [7]. Amlodipine besylate was determined by spectrophotometric [8-10], Spectrofluorometric [11], LC [1], RP-HPLC [12-14], LC-MS [15], HPTLC [16] and HPLC stability indicating assay method [17]. On the other hand, binary mixture of Amlodipine besylate and Perindopril Erbumine was determined by RP-HPLC [3]. To our knowledge there is no analytical method has been published for determination of Perindopril in the form of L-arginine salt in its combination with AB.

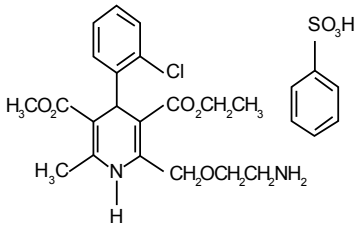

Figure 1: Chemical structure of Amlodipine Besylate.

Amlodipine besylate and PA are co-formulated together in COVERAM® tablets which are indicated as substitution therapy for the treatment of hypertension and/or stable coronary heart disease in patients already controlled with separate doses of perindopril and Amlodipine. The present work describes validated TLC and RP-HPLC methods for simultaneous determination of AB and PA in their bulk powders and tablet dosage forms. The advantage of the proposed methods is that AB and PA can be determined on a single chromatographic system with the same detection wavelength with high sensitivity. The developed RP-HPLC method has an additional advantage that can be applied for determination of the proposed drugs in plasma in therapeutic drug monitoring studies because of its high sensitivity and suitable R_f values of the separated peaks to their in vitro analysis.

***Corresponding author:** Marco M. Zaki, Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Beni-Suef University, Alshaheed Shehata Ahmed Hegazy st. 62514, Beni-Suef, Egypt, Tel: 01227027144; E-mail: marcomounir11@yahoo.com

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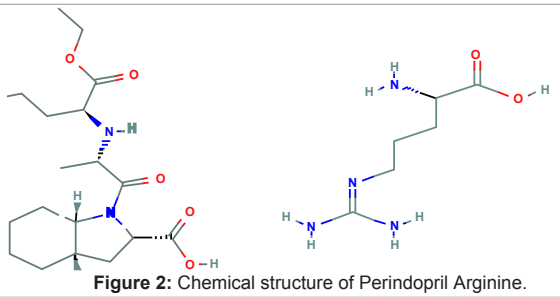

Figure 2: Chemical structure of Perindopril Arginine.

Experimental

Materials

Pure standards: 1- Amlodipine Besylate was kindly supplied by Hikma Pharma S.A.E., 6th of October- Egypt. Its purity was reported to be 99.5% according to the company certificates.

2- Perindopril Arginine was kindly supplied by by Servier (Ireland) industries Ltd. for Les Laboratories Servier-France. Its purity reported to be 99% according to the company certificates.

Pharmaceutical formulation: 1- Two COVERAM® tablet dosage forms, labeled COVERAM® 5/10 (B.N. 73935), labeled to contain 5 mg and 10 mg of PA and AB, respectively. COVERAM® 10/5 (B.N. 73934), labeled to contain 10 mg and 5 mg of PA and AB, respectively. Manufactured by Servier (Ireland) industries Ltd. for Les Laboratories Servier-France.

Chemicals and solvents: All chemicals and solvents used throughout this work were of analytical grade and were used without further purification

1- Chloroform, glacial acetic acid, pottasium dihydrogen phosphate all were from (El NASR Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).

2- Acetonitrile, methanol and triethylamine are of HPLC grade (SDS, France).

3- Deionized water (SEDICO pharmaceuticals Co., Cairo, Egypt).

Instrumentation

1- Sample applicator for TLC linomat V with 100 µL syringe (Camage, Muttenz, Switzerland).

2- TLC Scanner 3 densitometer (Camage, Muttenz, Switzerland). Controlled by WIN CATS software (V 3.15, Camage).

The following requirements are taken into consideration:

- Slite dimensions=3.00×0.45,

- Scanning speed=20 mms^{-1},

- Data resolution=100 $\mu.step^{-1}$.

3- TLC plates (20 cm×10 cm) coated with silica gel 60 F_{254} (Merck, Germany) with 0.2 mm thickness.

4-UV lamp with short wavelength 254 nm (VL-6.LC, MARNE LA VALLEE cedex 1, FRANCE) was used for scanning until optimization of the proposed method.

5-Shimadzu 2010C integrated HPLC equipped with quaternary gradient pump, 2010C UV-VIS detector, 2010C column oven and 2010C programmable auto sampler controlled by CLASS-VP software. Eclipse XDB C18 column 25 cm (4.6 mm×5 µm) was used as a stationary phase.

Prepared solutions

Stock standard solutions (1000 µg.mL^{-1}): Stock standard solutions of AB and PA were prepared by weighing accurately and separately 0.1 g of pure powder of each into two separate 100 mL volumetric flasks, 75 mL methanol was added, shaking well and then the volume was completed to the mark with methanol.

Working standard solutions (100 µg.mL^{-1}): Working standard solutions of AB and PA were prepared by accurately transferring 10 mL each of AB and PA from their respective stock standard solutions (1000 µg.mL^{-1}) into two separate 100 mL volumetric flasks, then completing to the mark with methanol (for TLC-densitometric method) or with phosphate buffer: acetonitrile (60:40, v/v) (for HPLC method) to get 100 µg.mL^{-1} working solution of each.

Laboratory prepared mixtures: Different laboratory prepared mixtures containing different ratios of AB & PA were prepared by accurately transferring different volumes of each drug from its respective working standard solutions (100 µg.mL^{1}) to 10 mL volumetric flasks and then completing the volume using the suitable solvent.

Procedures

Linearity and construction of calibration curves: 4.4.1.1 TLC-densitometric method: Accurately measured aliquots equivalent to (1-10 µL) each of AB & PA were separately transferred from their respective stock standard solutions (1000 µg.mL^{-1}) and were applied in triplicates on TLC aluminum plates (20×10 cm), prewashed with methanol and preactivated at 100°C for 15 minutes. Samples were applied as bands using the Camage TLC sampler. The band length was 4 mm and the bands were applied 15 mm from the bottom edge of the plate. Ascending development was performed in a chromatographic tank previously saturated for an hour with chloroform:methanol:deionized water:glacial acetic acid:triethylamine (10:7:5:0.3:0.2, by volume). The migration distance was 80 mm from the lower edge and the developed plates were then air dried. AB and PA bands were scanned at 208 nm and the calibration curves were constructed by plotting the mean integrated peak area versus the corresponding concentrations from which the regression equations were computed.

HPLC method: Accurately measured aliquots equivalent to (10-500 µg) each of AB and PA were separately transferred from their respective stock standard solution (1000 µg.mL^{-1}) into two series of 10 mL volumetric flasks, and then completed to the volume with the mobile phase. Triplicate 20 µL injections were made for each concentration maintaining the flow rate at 1 mL.min^{-1} and the effluent was UV-scanned at 210 nm. The chromatographic separation was performed on C18 column using phosphate buffer:acetonitrile (60:40 v/v) pH 4.6 as a mobile phase. The peak areas of AB and PA were recorded and calibration curves relating the obtained peak areas to corresponding concentrations were constructed.

Analysis of laboratory prepared mixtures: Synthetic mixtures containing of AB and PA in different ratios were analyzed following the procedure under linearity and construction of calibration curves for each method.

Application to pharmaceutical formulation: The content of 20 tablets each of COVERAM® (5/10) & (10/5) tablets was separately powdered and mixed well. An amount of each powdered tablets equivalent to 100 mg of AB (and the corresponding amount of PA)

was accurately and separately weighed and transferred to 100 mL volumetric flask, 75 mL methanol was added and the prepared solutions were magnetically stirred for about 30 min. The solutions were then cooled well; the volume was then completed with methanol to get 1000 µg.mL^{-1} stock solution of each dosage form and then filtered. Appropriate dilutions were made to prepare 100 µg.mL^{-1} working solution of each dosage form and the procedure detailed under linearity and construction of calibration curves for each method was followed. The concentrations of AB & PA were calculated from the corresponding regression equations.

Results and Discussion

Method developments and optimization

TLC-densitometric method: This technique offers a simple way to quantify directly on TLC plates by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing the peak area of the unknown band to a standard curve from reference materials chromatographed under the same condition [18]. The developed TLC- densitometric method depends on the difference in R_f values of AB (R_f=0.21) and PA (R_f=0.31) (Figure 3).

Parameters affecting the method performance were tested in order to obtain maximum chromatographic separation, such as developing system, scanning wavelength and slide dimension. In order to choose the optimum developing system, methanol: chloroform (5:5, v/v) was firstly used. Unfortunately, bad resolution among the studied drugs with tailed peaks was obtained, the second step is to change their ratio (7:3), (3:7), (9:1) & (2:8) but with no improvement. Addition of acetic acid to the developing system with different ratios was then tested which significantly enhanced the resolution but with small R_f value for AB and asymmetric peaks for AB and PA. TEA was then tested in different ratios in order to minimize the peaks tailing and finally water was added to the system to improve the R_f values of the separated drugs. Good separation was obtained when chloroform:methanol:deionized water:glacial acetic acid:triethylamine (10:7:5:0.3:0.2, by volume) was used as a developing system. Different band dimensions were tested in order to obtain sharp and symmetrical peaks. The optimum band width chosen was 4 mm and interspace between bands was 8.9 mm. Different scanning wavelengths (208, 210, 212 and 254 nm) were tested, but the best sensitivity was obtained when AB and PA were scanned at 208 nm. The slit dimensions of scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference from the adjacent bands. Different slit dimensions were tried where 5 mm×0.2 mm proved to be the slit dimension of choice which provides the highest sensitivity.

RP-HPLC method: A validated isocratic RP-HPLC method with UV detection was developed for the simultaneous quantitation of AB and PA. It depends on the chromatographic separation of the two drugs using C18 column and a mixture of buffer: acetonitrile (60:40, v/v) as a mobile phase with UV detection at 210 nm. All of the experimental conditions affecting the method performance were investigated. Chromatographic separation was started using phosphate buffer:acetonitrile (70:30) pH=5 as a mobile phase but bad resolution was obtained. Then changing the ratio of organic modifier was tested (20-50%), it was found that using acetonitrile more than 40% resulted in bad Chromatographic separation and short analysis time. On the other hand decreasing acetonitrile ratio in the mobile phase (less than 40%) did not affect the resolution but decrease the analysis time. Also phosphate buffer with different pH values was tested (3-6) where the optimum separation with symmetric untailed peaks was obtained when using pH=4.6. The effect of the mobile phase flow rate (1, 1.5 and 2 mL.min^{-1}) on the separation was also tested, where using flow rate of 1 mL.min^{-1} gave the optimum chromatographic separation within reasonable time analysis. Scanning wavelength (210, 220 and 254 nm) was tested in order to improve the sensitivity of the developed method. Where scanning at 210 nm gave the best sensitivity. Finally,

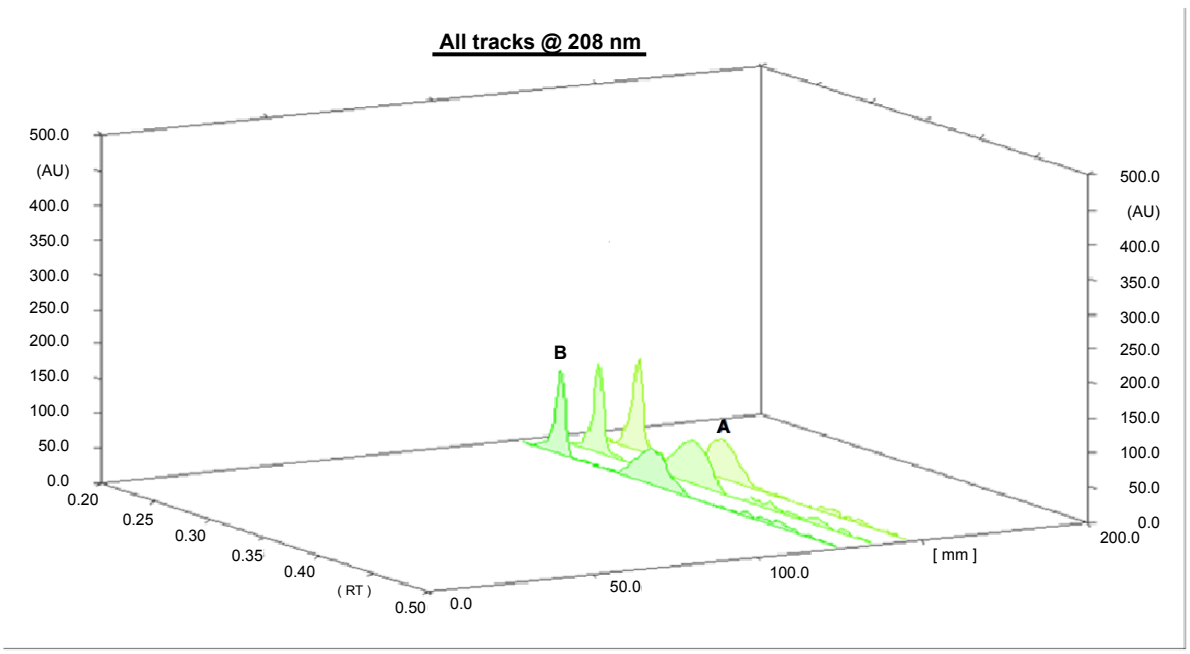

Figure 3: Thin layer chromatogram of mixture of (A) of Amlodipine besylate (B) Perindopril Arginine, using chloroform:methanol:deionized water:glacial acetic acid:triethylamine (10:7:5:0.3:0.2, by volume).

a satisfactory separation was obtained by using mixture of phosphate buffer:acetonitrile in the ratio of (60:40) pH=4.6 as a mobile phase, maintaining the flow rate at 1 mL min^{-1} with UV detection at 210 nm, where PA was separated after 4.8 min and AB after 7.3 min, and no interference was found among the two peaks. Typical chromatogram is shown in Figure 4.

Method validation

Validation of the proposed method was performed according to ICH guidelines [18,19].

Linearity and range: The linearity of the proposed methods was evaluated and it was evident in the range of 1-10 µg/band (for both AB and PA in the TLC densitometric method) and 1-50 $\mu g.mL^{-1}$ (for RP-HPLC method).

The regression equations were calculated and found to be: -

For the TLC densitometric method

R_1:- Y=318.94X + 912.57, R=0.99992

R_2:- Y=363.25X + 51.695, R=0.99991.

Where Y is the peak area, X the concentration in µg $band^{-1}$ and R is the correlation coefficient (Table1).

For RP-HPLC method

R_3:- Y=47662x−15018, R=0.99995.

R_4:- Y=10284x−1490.2, R=0.99994.

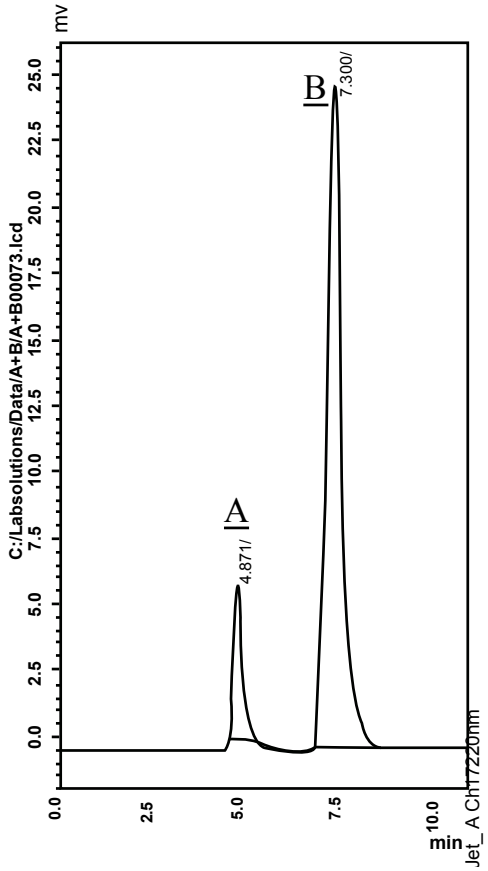

Figure 4: HPLC chromatogram of the mixture of (a) Amlodipine besylate (b) Perindopril Arginine at 210 nm using phosphate buffer: acetonitrile (60:40, v/v)..

Parameters	TLC		HPLC	
	AB	PA	AB	PA
Linearity range	1-10 µg.band^{-1}	1-10 µg. band^{-1}	1-50 µg.mL^{-1}	1-50 µg.mL^{-1}
Slope	318.94	636.25	47662	10284
Intercept	912.57	51.695	-15018	-1490.2
Correlation coeff	0.9998	0.9998	0.99995	0.99994
Accuracy	100.03%	99.70%	99.70%	100.05%
Precision *Repeatability*	0.92	0.92	1.13	0.212
Intermediate precision	1.05	0.650	0.707	0.849

Table 1: Analytical parameter of the proposed methods for determination of AB and PA.

Where Y is the peak area (of AB and PA), X is the concentration in $\mu g.mL^{-1}$ and R is the correlation coefficient (Table 1).

Precision

Repeatability: Three concentrations of AB and PA (4, 6 and 8 µg $band^{-1}$) for TLC-densitometric method (10, 20 and 30 $\mu g.mL^{-1}$) for RP-HPLC method were analyzed three times intradaily using the proposed methods. Good %RSD values were obtained; Table 1 confirmed the repeatability of the methods.

Intermediate precision: The previous procedures were repeated interdaily on three different days, results given in Table1 confirmed Intermediate precision of the developed methods.

Accuracy: Accuracy of the proposed methods was checked by applying the proposed methods for determination of pure samples of the studied drugs. The concentrations were calculated from the corresponding regression equations and the results are shown in Table 1. Accuracy was further assessed by applying the standard addition technique on Coveram® tablets where good recoveries were obtained revealing no interference from excipients and good accuracy of the proposed methods (Table 2).

Selectivity: Selectivity of the proposed methods is evident from the TLC and HPLC chromatograms in Figure 3 and Figure 4, respectively. Also specificity of the methods was proved from the good recovery percentages obtained when they were applied for determination of laboratory prepared mixtures (Table 2). The proposed methods were applied for determination of AB and PA in laboratory prepared mixtures and the results are shown in Table 2.

Robustness: Robustness of the proposed methods was evaluated in the development phase by making small changes in the composition of mobile phase and detection wavelength. The low value of %RSD shows that the method is robust and that deliberate small changes in the studied factors did not lead to a significant change in retention values, area or symmetry of the peaks.

System suitability testing for HPLC: System suitability testing parameters for TLC densitometric and RP-HPLC methods is based on the concept that the equipment, electronics, analytical operations and samples constitute an integrated system that can be evaluated as a whole. System suitability parameters are given in Table 3.

Conclusion

This work provides accurate, precise and sensitive chromatographic methods for determination of the studied mixtures without preliminary separation steps. The developed TLC–densitometric method achieved high resolution with less time consuming and cost effective while the

		TLC		HPLC	
		AB	PA	AB	PA
L.P.M		99.47 ± 1.87	99.98 ± 1.12	99.94 ± 1.46	99.52 ± 1.45
COVERAM®[b] 5/10 B.No.73935	% Recovery	100.93% ± 3.181	99.11% ± 2.687	100.71% ± 2.687	100.20% ± 1.131
	St addition[a]	99.33 ± 1.53	98.67 ± 2.08	99.77 ± 1.97	100.3 ± 0.82
	Degree of freedom	8	8		
	F-TEST[c] (5.050)	1.869	4.892		
	Student T-test[c] (2.228)	0.162	1.255		
COVERAM® 10/5 B.No.73934	% Recovery	99.92% ± 0.640	99.695% ±1.088	100.995% ± 1.421	99.856% ± 1.63
	St addition[a]	99.33 ± 1.53	98.67 ± 2.08	100.3% ± 2.141	99.21% ± 2.43
	Degree of freedom	10	10		
	F-TEST[c] (3.728)	2.958	1.342		
	Student T-test[c] (2.306)	0.200	0.200		

[a] Average of 6 determinations.
[b] Average of 5 determinations.
[c] The values in the parenthesis are corresponding theoretical value at degree of freedom p=0.05

Table 2: Determination of AB and PA in laboratory prepared mixtures and Coveram® tablet by the proposed methods and statistical comparison with each other

SST	TLC		HPLC	
	AB	PA	AB	PA
Resolution(R_s)	2		5.55	
Selectivity factor(α	1.4		2.34	
Capacity factor(K')	4	5.6	1.45	0.62
Tailing factor	0.9	1.04	1.16	1.1
N(Number of plates)			3504.6	2361.96
HETP			0.007	0.010

Table 3: System suitability testing parameter of TLC-densitometric and HPLC methods.

RP-HPLC method developed is more specific.

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