Original Research Article

Determination of Amlodipine Besilate and Azilsartan Medoxomil by UHPLC, HPTLC and Spectrophotometric techniques

ABSTRACT

Aims: To develop methods with complete validation according to ICH guidelines and to be applied for the determination of both drugs in laboratory prepared mixtures and in synthetic tablets.

Study design: Ultra high performance liquid chromatography (UHPLC), High performance thin layer chromatography (HPTLC) and visible spectrophotometric methods are developed for determination of amlodipine besilate and azilsartan medoxomil in laboratory-prepared mixtures and in synthetic tablets.

Methodology: Two techniques have been developed for the simultaneous determination of amlodipine besilate and azilsartan medoxomil in pure form and synthetic tablets. The first was UHPLC in which separation was achieved on a C18 column using 0.1% *o*-phosphoric acid - acetonitrile - methanol (60:10:30, by volume) as mobile phase with detection at 243nm. The second was HPTLC where separation was performed on silica gel 60 F254 plates using chloroform- tolune-methanol-glacial acetic acid (7: 1.5: 1.5: 0.5 by volume) as a developing system and UV detection at 243nm. In addition, visible- spectrophotometric method was developed for determination of amlodipine besilate in presence of azilsartan medoxomil through formation of yellowish orange colored product after reaction of amlodipine besilate with anisaldehde in acid medium with λ_{max} at 443 nm.

Results: UHPLC method was linear over the concentration ranges of 2-20 µg/ mL and 4-40 µg/ mL while HPTLC method was linear over the concentration ranges of 0.2 -4.0 µg/ spot and 0.5-8.0 µg/ spot for amlodipine besilate and azilsartan medoxomil, respectively. The visible spectrophotometric method was found to be valid over the concentration range of 10–80 µg/mL for amlodipine besilate.

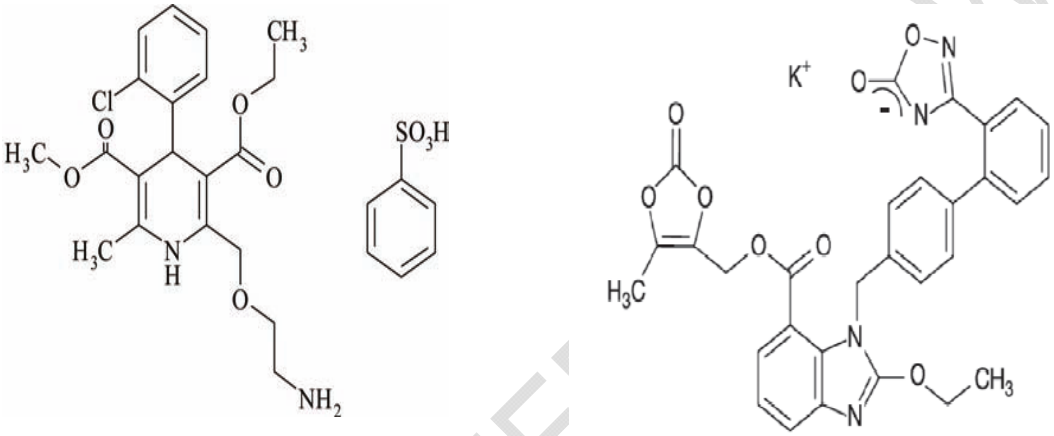
Conclusion: The proposed three techniques are rapid, accurate and precise, thus can be effectively applied for the routine estimation of both drugs in bulk and in their combined formulations.

Keywords: Amlodipine besilate; Azilsartan medoxomil; UHPLC; HPTLC; visible-spectrophotometry

1. INTRODUCTION

Amlodipine besilate (ALD-B); 3-ethyl 5-methyl-2-[(-2- (aminoethoxymethyl]-4-(2-chlorophenyl)-1,4-dihydro6- methyl-3,5-pyridinedicarboxylate is a potent di hydropyridine calcium channel blocker while Azilsartan medoxomil (AST-M); (5 – Methyl – 2 – oxo -1,3 – dioxol -4 – yl) methyl 2 – ethoxy -1 – {[2'- (5 - oxo -4,5 – dihydro - 1, 2, 4 – oxadiazol -3 - yl) biphenyl – 4 – yl] methyl } - 1H – benzimidazole -7 - carboxylate monopotassium salt is a

potent angiotensin II receptor blocker[1]. Both drugs used in treatment of hypertension. A number of HPLC [2-7], HPTLC[8-10], LC/MS[11,12], UV-Vis spectrophotometric[13-16] and fluorometric[17-19] methods were reported for the quantification of Amlodipine besilate and Azilsartan medoxomil alone and in combination with other drugs. Meanwhile, few HPLC [20,21] were reported for the simultaneous determination of Amlodipine besilate and Azilsartan medoxomil in combination. The reported method [20] involved RP-HPLC method for simultaneous estimation of ALD-B and AST-M in tablet dosage form using phenomenex luna ODSC18 column with UV detection at 254 nm, a mobile phase of phosphate buffer pH 2.5 adjusted with O-phosphoric acid: acetonitrile (60: 40 v/v), at flow rate of 0.7 mL / min and retention times were 5.918 min and 14.901 min for ALD-B and AST-M, respectively. Thus the objective of the present study is to develop simple and accurate methods for determination of this combination in solid dosage form.



Amlodipine besilate Azilsartan medoxomil

Fig.1: Chemical structure of Amlodipine besilate and Azilsartan medoxomil.

2. EXPERIMENTAL

1.1. Instrumentation

- The UHPLC system used was an Agilent 1100 UPLC with binary pump and UV detector, analysis was performed on a Kinetex C 18 column (100 mm, 4.6 mm i.d., 2.6 µm); Torrance, USA.
- Merck TLC plates used were 20 x 20 cm precoated with silicagel 60 F 254 (Flukachemie, Switzerland), a camag Linomate 5 sample applicator equipped with a 100 µL syringe (Hamilton, Germany) 20 x 20 cm twin through glass chamber (Camag). The plates were scanned with a camag TLC scanner 3 with WINCATS computer software (Switzerland) using UV lamp with short wavelength (254 nm) (Desega- Germany).
- Shimadzu UV/Vis spectrophotometer (PC – 1601, Tokyo, Japan), using 1.0 cm quartz cells. Scans were carried out in the range from 200–400 nm at 0.5 nm intervals. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software.

2.2. Materials and Reagents

Pure samples: Amlodipine besilate and Azilsartan medoxomil were kindly supplied by RAMEDA Co., Giza, Egypt, and their purity were 99.82% and 99.77%, respectively as stated by the supplier. Zacras® LD and HD tablets (Takeda, Japan, cannot be obtained). Magnesium stearate (ADWIC, Qalyubia, Egypt). Avicel (NF 18/USP23 M 101, Tong Sing Chemicals Co., Taipei, Taiwan). Anisaldehde (Sigma, Schnelldorf, Germany), 5% and $4x10^{-2}$ M solutions in methanol, the later was prepared by dissolving 0.46 mL in methanol to obtain 100 mL.

Solvents: Tolune, acetone, hydrochloric acid, nitric acid, sulfuric acid and glacial acetic acid were obtained from El-Nasr Co., Qalyubia, Egypt. Chloroform, methanol, ethanol and O-Phosphoric acid were obtained from Sigma Aldrich (Schnelldorf, Germany) and Acetonitrile HPLC grade was obtained from Fisher (Loughborough, UK).

Preparation of Standard solutions

- Standard stock solution of ALD-B and AST-M were prepared as 1 mg mL^{-1} in methanol. Working solutions were freshly prepared by suitable dilution of each stock solution with methanol to obtain a concentration of 0.5 mg/mL or 0.1 mg/mL from each drug.
- $4x10^{-2}$ M ALD-B solution was prepared by dissolving 1.636 g drug to make 100 mL in methanol.

Synthetic tablets

They were prepared by mixing 20 mg of AST-M, 2.5 mg of ALD-B (low dose tablets) or 20 mg of AST-M, 5 mg of ALD-B (high dose tablet) with 1.05 mg magnesium stearate and completed to 150 mg with avicel.

2.3. Procedures

2.3.1. Linearity

i. **UHPLC method-** Aliquots of working standard drug solutions (0.1 mg /mL) containing 0.02-0.2 mg of ALD-B and 0.04-0.4 mg of AST-M were introduced into two separate series of 10- ml volumetric flasks and adjusted to the volume with methanol. Triplicate 10µLwere injected were made for each concentration on a C18 column followed by elution with a mobile phase of 0.1% O-phosphoric acid - acetonitrile - methanol (60:10:30, by volume) at a flow rate of 1 mL/ min with UV detection at 243 nm.. The peak area was then plotted against the corresponding drug concentration and regression equation was computed.

ii. **HPTLC method-** Different volumes of standard solution (1 mg/mL) containing 0.2-4.0 mg of ALD-B and 0.5-8.0 mg AST-M were introduced into two separate series of 10-ml volumetric flasks and adjusted to the volume with methanol. Ten µL from each solution were applied to pre-washed activated plates, as 6-mm bands, 6 mm apart, by means of a Camag Linomat IV automated spray-on band applicator equipped with a 100-µL syringe. The plates were developed with the mobile phase of chloroform-tolune-methanol-glacial acetic acid (6: 2.5: 1.5: 0.5 by volume) in a Camag twin-trough chamber previously saturated with mobile phase vapour for 20 min. Then plates were removed and air dried. Densitometry was performed at 243 nm in reflectance mode with slit dimensions of 6.00 mm × 0.3 mm and scanning speed of 20 mm/s. Peak area was then plotted against its corresponding drug concentration and regression equation was computed.

iii. **Visible spectrophotometric method-** Into a series of 20-mL test tubes, aliquots from standard ALD-B solution (0.5 mg mL^{-1}) in methanol equivalent to 0.1-0.8 mg were introduced. Then 3 mL of aqueous 1:1 H_2SO_4 and 2 mL of 5% anisaldehyde in methanol were added to each tube. The tubes were mixed and heated in a boiling

water bath for 20 min, cooled and transferred quantitatively into a series of 10-mL volumetric flasks. Volume was adjusted with methanol and absorbance of the developed yellow color was measured at 443 nm against a reagent blank.

2.3.2. Application to Synthetic tablets

Ten tablets of each low and high dose synthetic tablets prepared under "2.2. Material and reagents" were weighed accurately and finely powdered. Powder equivalent to 100 mg AST-M and 12.5 mg ALD-B or 100 mg AST-M and 25 mg ALD-B for low or high dose tablets, respectively were dissolved in 30 mL methanol in two separate 100-mL volumetric flasks. Both solutions were sonicated for 20 min and then diluted to 100 mL with the same solvent to obtain a solutions containing 1 mg mL^{-1} of AST-M and 0.125 mg mL^{-1} of ALD-B or 1 mg mL^{-1} of AST-M and 0.25 mg mL^{-1} of ALD-B for the two dose tablets, respectively. Both tablets solutions were analyzed using the proposed UPLC, HPTLC and spectrophotometric techniques.

3. RESULTS AND DISCUSSION

UHPLC method–The chromatographic separation of AST-M and ALD-B were optimized. Different mobile phases in different ratios were studied, where best peak shape and adequate separation of the two drugs was obtained by using 0.1% O-phosphoric acid - acetonitrile - methanol (60:10:30, by volume). Different flow rates and wavelengths were tried; good resolution with most sensitive detector response was obtained at 243 nm using a flow rate of 1 mL min^{-1}. Under the described parameters, the peaks of the two drugs were well resolved at retention time of 2.259 and 4.474 for ALD-B and AST-M, respectively, as shown in Fig.(2).

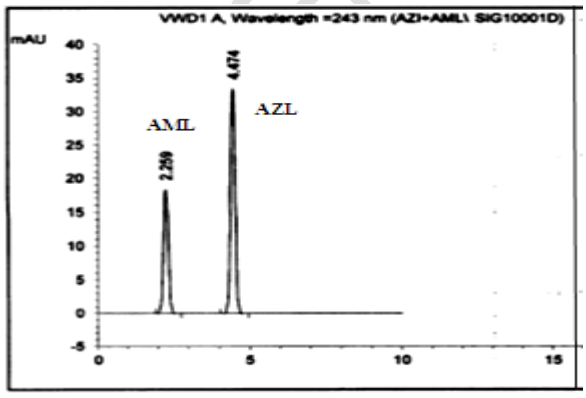


Fig. 2: UPLC chormatogram of Amlodipine besilate (2 µg/ mL) and Azilsartan medoxomil (8 µg/ mL).

HPTLC method-Different mobile phases in different ratios and at different λ_{max} for detection were tried. It was found that chloroform- tolune-methanol-glacial acetic acid (6: 2.5: 1.5: 0.5 by volume) as a developing system followed by densitometric determination at 243 nm offered best separation and resolution. Where R_f were 0.4 and 0.7 for ALD-B and AST-M, respectively, Fig.(3).

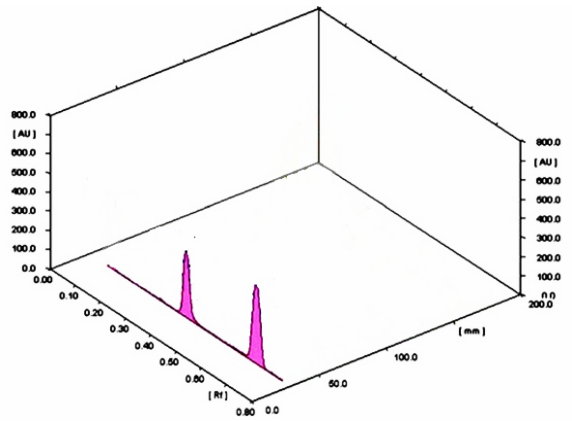


Fig. 3: Densitogram of Amlodipine besilate (3 µg/ spot) and Azilsartan medoxomil (3µg/ spot).

Visible spectrophotometric method- ALD-B contained primary amino group which can be allowed to condense with aldehydic groups in acid medium[22] thus the reaction of the drug with anisaldehde was studied in H_2SO_4 medium and found to produce yellowish- orange colored Schiff-base having maximum absorption at 443 nm; Fig.(4).

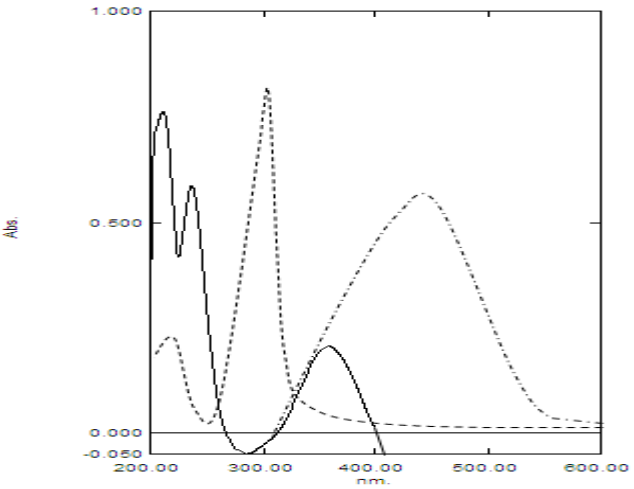


Fig. 4: Absorption spectra of 20 µg mL^{-1} Amlodipine besilate (–) , 50 µg mL^{-1} Amlodipine besilate -anisaldehde Schiff-base (-.-.-.) and reagent blank (….).

The reaction conditions were optimized as follow:

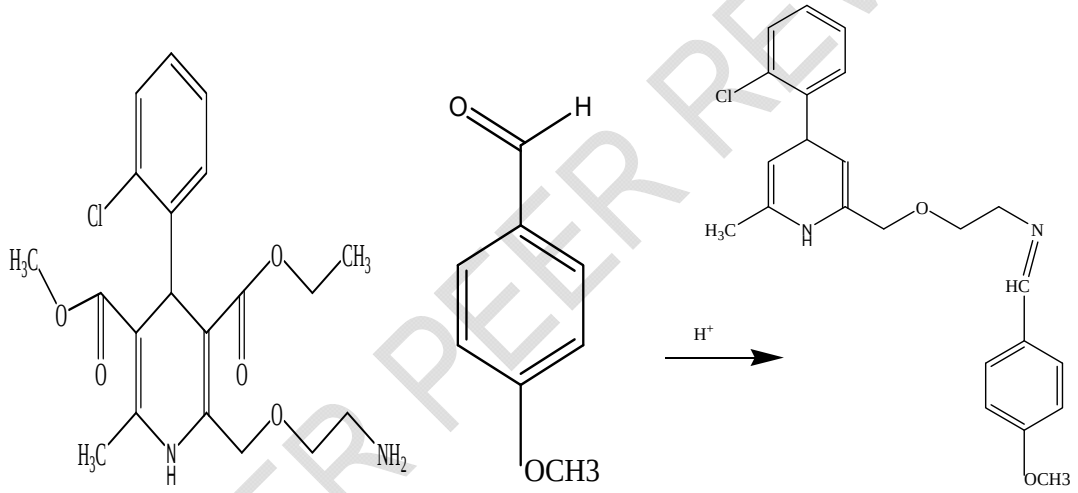
- **Effect of type of acid-** No reaction produced upon using HCL, nitric acid and acetic acid. The reaction was found to be produced only in presence of sulfuric acid, hence 1:1 H_2SO_4 was used.
- **Effect of volume of 1:1 sulfuric acid-** Different volumes (0.5-4.0 mL) of 1:1 sulfuric acid in water were allowed to react with definite concentration of drug. Where 2.5 to

3.5 mL of 1:1 sulfuric acid was found to be sufficient for maximum sensitivity at the relevant maxima, thus 3 mL of 1:1 H_2SO_4 was used throughout the procedure.

- **Effect of anisaldehde volume-** Different volumes (0.5-3.0 mL) of 5% anisaldehde were allowed to react with definite concentration of drug. Where 1.5 to 2.5 mL of 5% anisaldehde gave maximum intensity at 443 nm, thus 2 mL of 5% anisaldehde was used throughout the procedure.
- **Effect of temperature and heating time-** The reaction of ALD-B with anisaldehde was carried out using different temperature (50-100°C). Maximum absorbance was attained after 20 min at 100°C and the colour remained stable for further 2 hours.
- **Effect of diluting solvent-** water, ethanol, acetonitrile, acetone or methanol was tried as diluting solvent for the reaction mixture, where methanol gave the highest sensitivity.

Stoichiometry of the reaction

Job's method[23] was applied using $4x10^{-2}$ M solutions of AMD-B and anisaldehde. A ratio of 1:1 between the drug and anisaldehde in H_2SO_4 medium was obtained due to presesnce of a free amino group were suggesting the following mechanism:



Amlodipine anisaldehyde Schiff base

Scheme (1): The suggested reaction mechanism of Amlodipine with anisaldehyde.

The final reaction product was confirmed by IR [24] where the spectrum of pure ALD-B showed two peaks at 3301 and 3156 cm^{-1} corresponding to primary amino group and two characteristic peaks at 1695 and 1677 cm^{-1} due to presence of two carbonyl groups of ester linkage; Fig.(5a), while IR spectrum of final reaction product showed disappearance of primary amine peaks indicating that aldehyde group of anisaldehde reacted with primary amine of ALD-B and formation of Schiff base which also showed disappearance of two peaks of two $C=O^-$ of ester linkage in ALD-B; Fig.(5b). This disappearance is due to heating with 1:1 H_2SO_4 that cause hydrolysis of two ester groups to their corresponding

carboxylic acid followed by decarboxylation and this was confirmed by IR spectral analysis of the drug with 1:1 H_2SO_4; Fig.(5c).

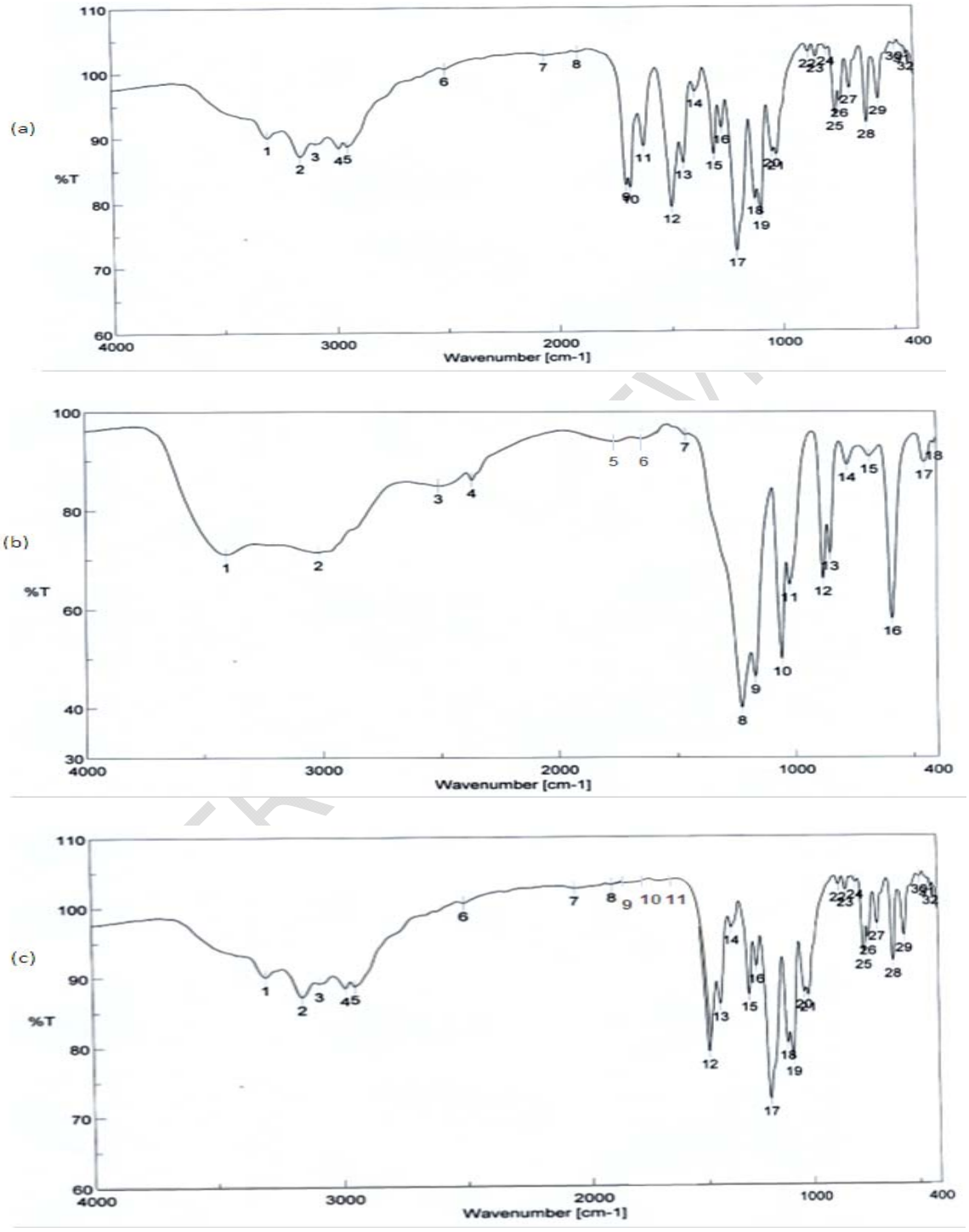


Fig. 5: IR spectrum of: a) amilodipine besilate, b) amilodipine-anisaldehde product and c) amilodipine in 1:1 H_2SO_4.

Method Validation

- **System suitability-** System suitability test was performed in accordance with USP[25] to ensure system performance before or during the drug analysis. Results shown in Table 1 indicate adequate resolution

Table 1: System suitability results of the UPLC method.

Parameter	ALD-B	AST-M	Reference value
Number of theoretical plates (N)	6855	7033	The higher the value, the more efficient the column is
Resolution factor	14.95		>2
Capacity factor (K)	2.81	3.25	1–10
Selectivity factor	7.85		≥1

- **Linearity**-Under the described experimental conditions, linear calibration curves between peak areas to respective drug concentration were obtained through the concentration ranges of 2-20 µg/ mL and 4-40 µg/ mL by UHPLC method and 0.2 -4.0 µg/ spot and 0.5-8.0 µg/ spot by HPTLC method for ALD-B and AST-M, respectively. The visible spectrophotometric method was found to be valid over the concentration range of 10–80 µg/mL ALD-B. Regression parameters were computed and presented in Tables 2, where coefficient of determination ranged between 0.9992-0.9999.
- **Accuracy and precision-** Accuracy calculated as (R%) ranged from 99.52 to 101.05% for the two drugs. While intraday precision (RSD %) ranged from 0.18 to 2.11%, while intermediate precision ranged from 0.26 to 2.03% for both drugs; indicating good repeatability and reproducibility of the methods, Tables 2**.**
- **Selectivity**-It was determined by applying the proposed methods to synthetic prepared mixtures containing different ratio of the two drugs. Good mean % recoveries of 100.56±1.43 and 100.96±1.61were obtained for ALD-B and AST-M, respectively in UPLC method. While for HPTLC, % recoveries amounted to 101.07±0.88 and 100.06±1.08 for the two drugs, respectively. While for visible spectrophotometric method, the mean recoveries were 100.65 % ± 0.79 for ALD-B, (Tables 3 &4).

It is noteworthy to mention that the ratio of ALD-B: AST-M in the market preparation (Zacras® LD and HD tablets) is 1:4 and 1:8, respectively and ALD-B was selectively determined in presence of AST-M without any interference.

Application to synthetic tablets

The proposed methods were successfully applied for analysis of both drugs in the laboratory prepared tablets. The validity of the proposed method was further assessed by applying the standard addition technique. The results obtained were reproducible with acceptable SD (0.44-1.83), Tables (5&6). Statistical analysis of the results obtained by the proposed methods compared with a reported one [20] showed that the calculated t and F values are less than the tabulated ones indicating no significant difference between them confirming accuracy and precision at 95% confidence limit, Tables (5&6). However the two chromatographic proposed methods are more sensitive, less time and solvent consuming. The visible spectrophotometric method is more simple and selective for ALD-B without any interference from AST-M. Therefore, should be cost-effective for routine analysis in the pharmaceutical industry.

Table 2: Regression and validation parameters for the determination of amlodipine besilate and azilsartan medoxomil by the proposed methods.

	UPLC		HPTLC		Visible spectrophotometric method
	ALD-B	AST-M	ALD-B	AST-M	ALD-B
$\lambda_{max\ (nm)}$	243nm				443nm
Linearity range (µg mL^{-1})	2-20 µg/ mL	4-40 µg/ mL	0.2-4 µg/ spot	0.5-8 µg/ spot	10-80 µg/ mL
Regression parameters					
Slope (b)**± SD**	5.482± 0.0288	7.2348± 0.0607	3546.2± 31.3731	3697.7± 35.36	0.0102± 0.0075
Intercept (a)**± SD**	0.2279± 0.3858	0.7074± 1.4021	989.22± 70.4906	10.143± 113.37	0.0345± 0.0001
Correlation coefficient (r^2)	0.9999	0.9997	0.9997	0.9994	0.9992
Accuracy (R %)	99.76	99.75	99.69	99.52	101.05
Precision (RSD %)					
Intra day	1.71-2.11	0.18-1.33	0.71-1.81	0.71-1.81	0.49-1.51
Inter day (n=9)	1.57-1.90	0.26-1.33	0.36-2.03	1.42-2.03	0.83-1.52

Table 3: Determination of amlodipine besilate and azilsartan medoxomil in their synthetic mixtures by the proposed UHPLC and HPTLC methods

	UHPLC method				HPTLC			
Ratio ALD-B: AST-M	ALD-B added (µg/mL)	AST-M added (µg/mL)	% Recovery of ALD-B	% Recovery of AST-M	ALD-B added (µg/mL)	AST-M added (µg/mL)	% Recovery of ALD-B	% Recovery of AST-M
1:1	4	4	99.76	100.86	3	3	101.43	99.21
1:4	2	8	98.52	98.62	0.2	1.6	100.69	98.87
1:8	5	40	101.91	102.28	1	8	99.77	99.89
4:1	20	5	100.81	100.40	4	1	101.41	100.98
8:1	16	2	101.80	102.63	4	0.5	102.07	101.36
	Mean%±SD		100.56± 1.43	100.96± 1.61	Mean%±SD		101.07± 0.88	100.06± 1.08

Table 4: Determination of amlodipine besilate and azilsartan medoxomil in their synthetic mixtures by the proposed Visible spectrophotometric method

	Visible spectrophotometric method		
Ratio ALD-B: AST-M	ALD-B added (µg/mL)	AST-M added (g/mL)	% Recovery of ALD-B
1:1	50	50	100.33
1:4	10	40	101.49
1:8	10	80	101.11
4:1	40	10	99.46
8:1	80	10	100.87
	Mean%±SD		100.65±0.79

Table 5: Results obtained by the proposed UHPLC and HPTLC methods compared with reported method[20] for the determination of amlodipine besilate and azilsartan medoxomil in the synthetic tablets.

Parameter	UPLC		HPTLC		Reported method[20]	
	ALD-B	AST-M	ALD-B	AST-M	ALD-B	AST-M
	Low dose tablet		Low dose tablet		Low dose tablet	
Linearity	2-20	4-40	0.2-4	0.5-8	75-125	600-1000
N	5	5	5	5	5	5
Mean%±SD	101.21±1.01	101.07±1.00	101.13±1.16	101.03±1.32	100.68±0.97	99.89±1.06
Variance	1.02	1	1.35	1.74	0.94	1.12
t-	0.85	1.82	0.67	2.01	-	-
F-	1.08	1.12	1.43	1.55	-	-
Standard addition	101.60±1.35	100.81±0.44	100.34±1.47	100.81±1.83	-	-
	High dose tablet		High dose tablet		High dose tablet	
Linearity	2-20	4-40	0.2-4	0.5-8	75-125	600-1000
N	5	5	5	5	5	5
Mean%±SD	100.19±1.25	100.83±0.85	100.55±1.43	100.80±1.36	100.22±1.17	100.16±1.12
Variance	1.56	0.72	2.04	1.85	1.37	1.25
t-	0.04	1.07	0.40	0.81	-	-
F-	1.14	1.74	1.49	1.47	-	-
Standard addition	100.02±1.41	99.23±0.88	99.66±1.24	100.99±1.51	-	-

Table 6: Results obtained by the proposed Visible spectrophotometric method compared with reported method[20] for the determination of amlodipine besilate and azilsartan medoxomil in the synthetic tablets.

Parameter	Visible spectrophotometric method	Reported method[20]	
	ALD-B	ALD-B	AST-M
	Low dose tablet	Low dose tablet	
Linearity	10-80	75-125	600-1000
N	5	5	5
Mean%±SD	101.42±1.04	100.68±0.97	99.89±1.06
Variance	1.08	0.94	1.12
t-	1.17	-	-
F-	1.15	-	-
Standard addition	100.41±0.85	-	-
	High dose tablet	High dose tablet	
Linearity	10-80	75-125	600-1000
N	5	5	5
Mean%±SD	101.46±1.25	100.22±1.17	100.16±1.12
Variance	1.56	1.37	1.25
t-	1.63	-	-
F-	1.14	-	-
Standard addition	100.91±0.88	-	-

-The theortical t- and f- values at p= 0.05 were 2.31 and 6.39, respectively.

4. CONCLUSION

The proposed three techniques are rapid, accurate and precise, thus can be effectively applied for the routine estimation of ALD-B and AST-M in bulk and in their combined formulations. The sample recovery for all three methods was in good agreement with their respective label claims which suggested no interference of additives and excipients.

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