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SIMULTANEOUS QUANTITATIVE ANALYSIS OF OLMESARTAN MEDOXOMIL AND AMLODIPINE BESYLATE IN PLASMA BY HPTLC TECHNIQUE

Sapan K. Shah*, Dinesh P. Kawade, Alpana J. Asnani and Dinesh R. Chaple

Department of Pharmaceutical Chemistry, Priyadarshini J. L. College of Pharmacy, Electronic Zone Building, MIDC, Hingna Road, Nagpur-440016, Maharashtra, India.

***Corresponding Author: Sapan K. Shah**
Department of Pharmaceutical Chemistry, Priyadarshini J. L. College of Pharmacy, Electronic Zone Building, MIDC, Hingna Road, Nagpur-440016, Maharashtra, India.

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ABSTRACT

A rapid, simple and sensitive high-performance thin layer liquid chromatography (HPTLC) method has been developed for quantification of olmesartan medoxomil (OLM) and amlodipine besylate (AM) in plasma. The assay enables the measurement of OLM and AM for therapeutic drug monitoring with a minimum detectable limit of 2 ng mL^{-1}. The method involves simple, one-step extraction procedure and analytical recovery was above 85-98 %. The separation was performed on silica gel 60 F_{254}. The mobile phase was a mixture of chloroform: methanol: acetic acid in the ratio 7.5: 1.25: 0.2 *v/v/v* at optimum wavelength selected was 254 nm. The calibration curve for the determination of drugs in plasma was linear over the range 2-200 and 2-800 ng $spot^{-1}$ for AM and OLM. The coefficients of variation for inter-day and intra-day assay were found to be less than 15%. The method can be applied to pharmacokinetic study of OLM and AM in combined dosage form.

KEYWORDS: Olmesartan medoxomil, Amlodipine besylate, HPTLC, Plasma etc.

1. INTRODUCTION

Amlodipine; R,S-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-ethoxycarbonyl-5-methoxy-carbonyl-6-methyl-1,4-dihydropyridine (Figure 1), is a potent calcium channel blocker used in treatment of hypertension and angina pectoris.[1] Amlodipine is well absorbed following oral administration with peak blood concentrations occurring after 6–12 h. The bioavailability is about 60–65%. It has a prolonged terminal elimination half-life of 35–50 h and steady-state plasma concentrations are not achieved until after 7–8 days of administration. Amlodipine is extensively metabolized in the liver; metabolites are mostly excreted in urine together with less than 10% of a dose as unchanged drug.[2, 3] Several analytical methods for quantifying of amlodipine in biological fluids have been reported; such as capillary gas chromatography (GC) with electron capture detection[4, 5], GC with electron-impact mass spectrometry (EI-MS)[6, 7] and high-performance liquid chromatography (HPLC).[8–12] Several HPLC procedures have been also reported for the analyses of amlodipine based on MS–MS detection by using tandem mass spectrometry.[13–16] Olmesartan medoxomil; (5-methyl-2-oxo-1,3-dioxolen-4-yl)methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl)-phenyl]phenyl}methyl imidazol-5-carboxylate) (Figure 2), is a potent and selective angiotensin AT1 receptor blocker[17] which has been approved for the treatment of hypertension in the United States, Japan and European countries. The drug contains a medoxomil ester moiety and is cleaved rapidly by an endogenous esterase to release the active metabolite olmesartan.[18] Up to date, olmesartan has been determined in plasma and other biological fluids using high performance liquid chromatography (HPLC) coupled to fluorescent detection.[19, 20]

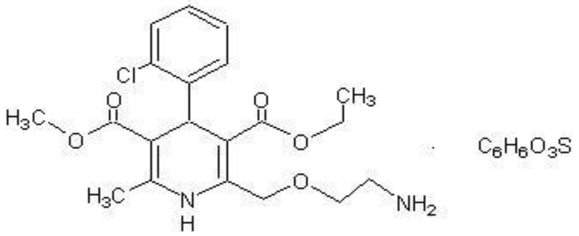

Figure 1: Structure of Amlodipine besylate

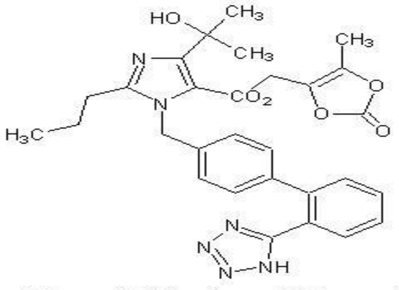

Figure 2: Structure of Olmesartan medoxomil

As per literature survey, no analytical methods have been reported for the simultaneous quantitative analysis of OLM and AM in biological fluids except HPLC.[21] The aim of our study was to develop a rapid, simple, accurate, precise, sensitive and reproducible HPTLC method, which can be utilize in pharmacokinetic research.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Olmesartan medoxomil (OLM) and Amlodipine besylate (AM) were kindly supplied by Ajanta Pharma Limited (Mumbai). All chemicals were analytical grade, chloroform; methanol and acetic acid (Merck, Mumbai, India) were used for developing TLC plates (Silica gel 60 F_{254}, Art 5554, Dc-Alufolien, Kieselgel 60 F_{254}, E.Merck, Darmstadt, Germany).

2.2 Instruments and chromatographic conditions

The samples were spotted in the form of bands 6 mm width with a Camag 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on silica gel precoated aluminum plate 60 F_{254}, (20 × 10 cm) with 250 µm thickness; (E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat IV autosampler (Switzerland). Linear ascending development was carried out in 20 cm × 10 cm or 50 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature (25^{o}C ± 2) at relative humidity of 36% ± 5. The length of chromatogram run was 8 cm. Densitometric scanning was performed on Camag HPTLC scanner III in the reflectance-absorbance mode at 254 nm operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp at 254 nm emitting continuous UV spectrum.

2.3 Selection of mobile phase and optimization of HPTLC method

The HPTLC procedure was optimized with a view to develop a simultaneous assay method for AM and OLM. The mixed standard stock solution (1 mg/mL of AM and 4 mg/mL of OLM) spotted on to HPTLC plates and run in different solvent systems. Optimization of HPTLC method was very critical in this case as both drugs had opposite polarity. AM was highly polar and OLM was non-polar. Initially, toluene: ethyl acetate: methanol (5:4:1 *v/v/v*) was selected but AM due to polar nature did not moved from application position. Hence to increase the polarity of the system toluene volume was decreased and ethyl acetate increased, but still AM did not run. Hence, more polar system n-butanol: acetic acid: water in different ratios tried to get optimum R_f for AM and OLM. First, n-butanol: acetic acid: water (5: 1: 1) was tried; in this mobile phase AM spot was moved R_f 0.5) but the OLM spot was over-run due to high polarity of the system. Then, the water was replaced with methanol and n-butanol with chloroform; finally, the mobile phase consisting of chloroform: methanol: acetic acid in the ratio 7.5: 1.25: 0.2 *v/v/v* was found optimum. The R_f value 0.37 and 0.81 were observed for AM and OLM respectively. In order to reduce the neckless effect HPTLC chamber was saturated for 20 mins prior to development of plates. The mobile phase was run up to a distance of 8 cm; which takes approximately 20 min for complete development of the HPTLC plate. (Figure-3)

2.4 Preparation of standard stock solution

Standard stock solution of OLM and AM was prepared by dissolving 10 mg of drug in 10 mL of methanol to get concentration of 1 mg/mL. From this solution 1 mL was taken out and further diluted to 10 mL to get concentration of 0.1 mg/mL.

2.5 Biological samples

The prepared suspension containing AM (0.4 mg mL^{-1}) and OLM (1.6 mg mL^{-1}) in 0.2 % tween 80 were administered orally to albino wistar rats (180-220 g) at dose of 2 mg kg^{-1} (AM) and 8 mg kg^{-1} (OLM) body weight after overnight fasting. The blood samples were collected from the retrobulbar plexus into anticoagulant-treated polypropylene tubes from 0 hr to 12 hr after drug administration. Blood samples collected were centrifuged immediately to separate the plasma. The plasma samples collected were stored at -20 $^{\circ}$C. The plasma samples then processed for drug recovery via solvent extraction.

2.6 Samples preparation (Extraction Procedure)

To 1000 µL of plasma samples in a borosilicate glass tube were added 5 mL of HPLC grade acetonitrile. After vortex mixing for 10 min at room temperature the samples were centrifuged at 10500 rpm for 10 min. The upper organic layer was transferred to a glass container and evaporated inside vacuum oven at 40 $^{\circ}$C. The dry residue was dissolved in 1 mL of mobile phase. The mixture was sonicated well for 10 minute and 20 µL of this solution was injected into liquid chromatography.

2.7 Stability

Stability assessments' defined as the resistance to change in concentration of an analyte in plasma under specified conditions. The stability of OLM and AM in rat plasma was investigated by adding known amount of drug to blank plasma samples to give concentrations of 10, 200 and 500 ng mL^{-1}. These were stored at -20 $^{\circ}$C and aliquots taken for 1 and 4 weeks for analysis.

2.8 Calibration curve

Linearity of instrument standard response was determined for each compound with different concentration calibration standards (final concentration ranging from 2-200 ng/mL for AM and 2-800 ng/mL for OLM, with drug ration maintaining constant 1:4). Calibration curves were constructed by plotting individual analyte peak area ratio as compared to corresponding concentration and fitting these data in regression analysis.

The calibration curves were constructed by the weighted regression method (1/x) of peak area of OLM or AM vs. actual concentrations.

3. RESULT AND DISCUSSION

3.1 Method development

All the concentrated and dried extracts obtained by the above method were re-dissolved in 1 mL of Acetonitrile by vigorous vortex-mixing and 50 µL aliquots of the sample were spotted on TLC plates with help of a Camag Linomat IV autosampler. The TLC plates were developed (8 cm) in a glass chamber (25×25×12 cm), with chloroform-methanol-acetic acid (7.5:1.25:0.2, v/v/v) (5 cm) where the spots were clearly separated as concentrated zones. The chamber was saturated with solvent system before development and the TLC plates were dried completely by Camag TLC plate heater III after development in solvent system.

The spot of OLM and AM were visualized under UV (λmax 254 nm). The R_f value 0.37 and 0.81 were observed for AM and OLM respectively (Figure 3). Determination of AM and OLM was done by scanning of the TLC plates Camag HPTLC scanner III in the reflectance-absorbance mode at 254 nm, operated by CATS software (V 3.15, Camag). Chromatogram obtained from blank plasma, blank plasma spiked with drug and sample collected from rats are shown (Figure 4).

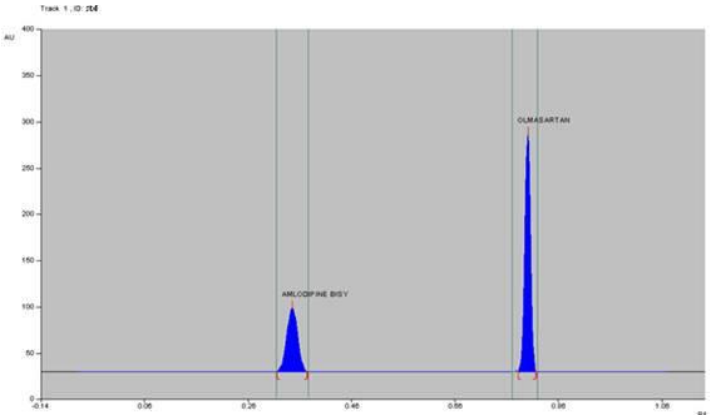

Figure 3: HPTLC chromatogram of laboratory mixture containing OLM 8 ng/mL and AM 2 ng/mL.

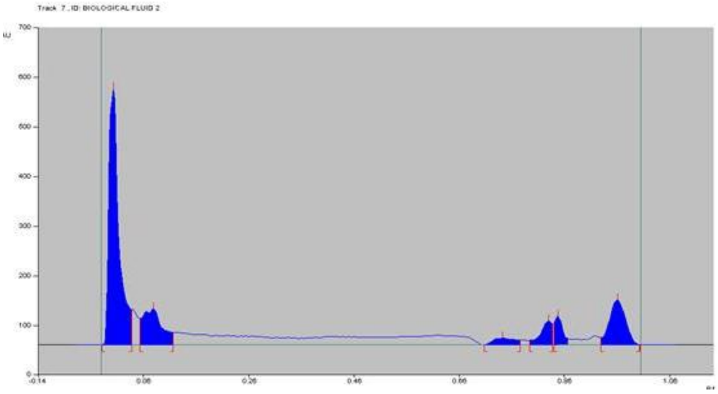

Figure 4(A): Blank plasma: Representative chromatograms of OLM and AM in wistar rat plasma.

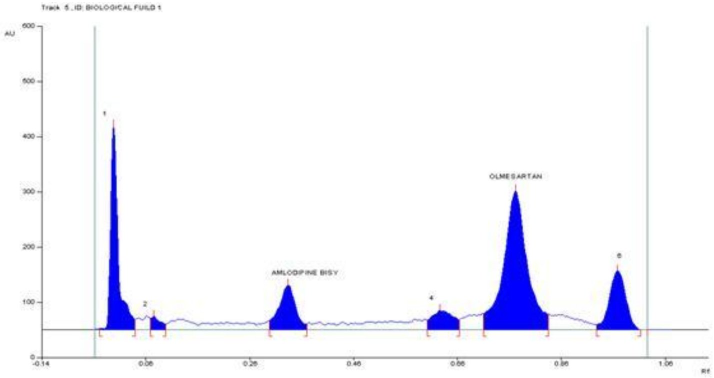

Figure 4(B): Plasma sample collected at 2.5 hrs :Representative chromatograms of OLM and AM in wistar rat plasma.

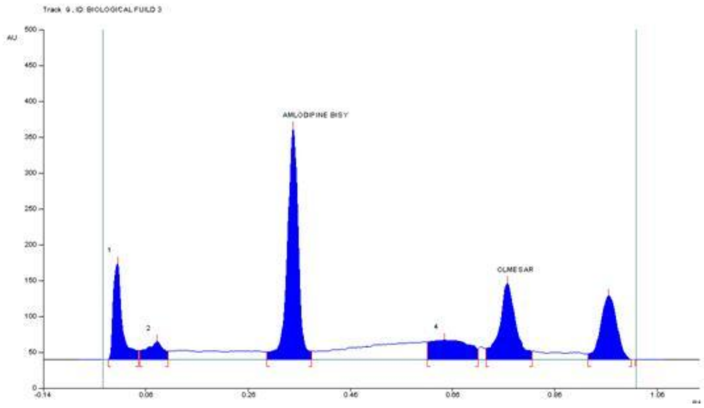

Figure 4(C): Plasma sample collected 6 hrs after, a single oral co-administration of OLM and AM in combination: Representative chromatograms of OLM and AM in wistar rat plasma.

3.2 Calibration curve

The calibration curve for the determination of OLM and AM in plasma was linear over the range 2-200 and 2-800 ng/mL AM and OLM. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (*R*) for calibration curves were equal to or better than 0.997. The relative standard deviation (R.S.D.) values of the slope were equal to or better than 5%. For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves.

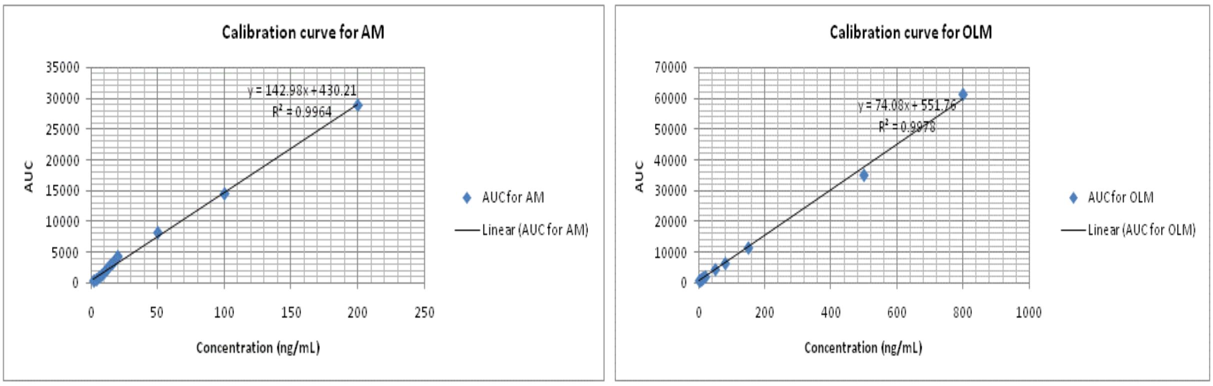

Figure 5: Calibration curve for AM and OLM in plasma

3.3 Recovery study

The relative analytical recovery for plasma at three different concentrations of OLM and AM was determined. Known amounts of drug were added to drug-free plasma in concentrations ranging from 2-800 ng/mL. The average recovery was in between 85-98 % for linear concentration range. The results are given in Table 1 and Table 2 for intraday and interday study respectively.

3.4 Stability study

Stability studies in plasma for OLM and AM, precision and accuracy of the analyte was < 15%. The stability tests indicated that OLM and AM are stable in rat plasma over the period of 1 month. Also, the stock solution remains stable for duration of 1 month when stored at 4°C express by the % variation < 2% for both OLM and AM.

Table 1: Recovery studies for OLM and AM (Intraday)

Sr. No.	Drug	Concentration added (ng/mL)	Peak area of standard	Mean peak area sample*	% Recovery*	±S.D.	C.V.
1	AM	2	6476.47	6147.87	94.92	3.145	3.31
	OLM	8	215.46	198.75	92.2	4.578	4.96
2	AM	20	21348	20987	98.3	10.48	10.66
	OLM	80	63891.54	60471.47	94.64	5.64	5.95
3	AM	200	21546.47	20145.89	93.49	2.57	2.74
	OLM	800	637457	608714.5	95.49	6.14	6.42

[*] Results are mean of three samples.

Table 2: Recovery studies for OLM and AM (Interday)

Sr. No.	Drug	Concentration added (ng/mL)	Peak area of standard	Mean peak area sample*	% Recovery*	±S.D.	C.V.
1	AM	2	6476.47	6217.87	96.00708	3.325	3.463
	OLM	8	215.46	208.75	96.88573	4.95	5.10
2	AM	20	21348	21007.12	98.40322	12.74	12.94
	OLM	80	63891.54	61571.47	96.36874	7.14	7.40
3	AM	200	21546.47	19145.89	88.85859	3.45	3.88
	OLM	800	637457	598714.5	93.92233	7.48	7.96

* Results are mean of three samples.

Table 3: Stability of OLM in rat plasma

Sr. No.	Sample concentration (ng/mL)	Concentration found (%)			±S.D.	Mean	CV	% Deviation (inaccuracy)
	Short-term stability for 24 h in plasma (-20˚C)							
1	10	101.45	104.67	98.78	2.949	101.6	9.38	1.6
2	200	109.56	115.68	96.58	9.753	107.3	11.2	7.3
3	500	89.65	84.68	87.69	2.503	87.34	7.11	-12.66
	Long-term stability for 1 month in plasma(-20˚C)							
1	10	102.59	98.41	87.69	7.685	96.23	7.47	-3.77
2	200	101.58	97.89	84.65	8.903	94.71	11.8	-5.29
3	500	87.52	81.62	115.24	17.95	94.79	8.22	-5.21

Table 4: Stability of AM in rat plasma

Sr. No.	sample concentration (ng/mL)	Concentration found (%)			±S.D.	Mean	CV	% Deviation (inaccuracy)
	Short-term stability for 24 h in plasma (-20˚C)							
1	10	98.78	87.56	99.54	6.708	95.29	8.38	-4.71
2	200	112.45	94.24	104.32	9.122	103.7	10.24	3.7
3	500	96.32	103.47	91.35	6.093	97.05	9.21	-2.95
	Long-term stability for 1 month in plasma(-20˚C)							
1	10	97.14	94.16	97.12	1.715	96.14	6.45	-3.86
2	200	116.48	110.67	101.52	7.542	109.6	13.8	9.6
3	500	91.79	87.136	94.78	3.852	91.24	8.22	-8.76

3.5 Limit of detection (LOD)

The limit of detection was defined, as the amlodipine concentration that produced a signal-to-noise ratio greater than 3. The limit of detection in plasma was 2 ng mL^{-1} for AM and 8 ng mL^{-1} based upon this criterion. At this level, the R.S.D. was lower than 15 %.

3.6 Accuracy and precision

Intraday and Interday assay performed to evaluate precision (% CV) and accuracy. The coefficient of variation was < 15% in rat plasma. The result suggests that within run and between run experiments the precision and accuracy for the analyte met the acceptance criteria.

4. CONCLUSION

The HPTLC method presented was direct, simple, selective, reproducible, sensitive and linear. The procedure was successfully applied to the simultaneous determination of the studied compounds in biological fluid without any interference from the additives and endogenous substances. The procedure was fully validated to meet the requirements of the food and drug administration center for drug evaluation and research (FDA/CDER) guidelines for bioanalytical method validation.[22, 23] This method was well suitable for routine application in the quality control laboratories and clinical laboratories because of the simplicity, economic, accuracy, sensitivity and reproducibility. The HPTLC method was applicable to pharmacokinetics studies of OLM and AM in rat. This method can be explore further to study the metabolism of OLM and AM[24] and establish drugs in other biological fluids like urine, CSF, peritoneal fluid, etc.

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