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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ATENOLOL AND AMLODIPINE BESYLATE IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

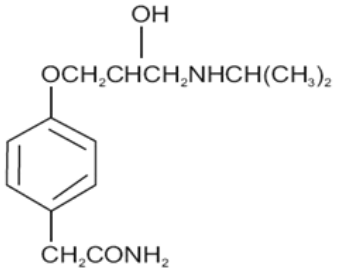
A new, simple, precise, rapid and accurate RP-HPLC (Reverse Phase – High Performance Liquid Chromatography) method has been developed for the simultaneous estimation of Atenolol (AT) and Amlodipine Besylate (AB) in bulk and in tablet formulations. The chromatographic separation was achieved on Agilent technologies 1200 series HPLC using Inertsil C_{18}, 5 µm, 250 mm x 4.6 mm column maintained at ambient temperature with mobile phase, Buffer: Acetonitrile: Methanol (4:3.5:2.5 v/v/v), flow rate 1.0 ml/min, load volume 10 µl and a run time of 10 min. The UV detection was performed at 225 nm. Buffer was prepared with Triethylamine and adjusted pH to 3.0 with Ortho-Phosphoric Acid. The retention time and mean recoveries obtained for AT was 2.23 min and 100.1%, for AB was 5.97 min and 100.4% respectively. Linearity response was established over the concentration range of 50-150 µg/ml for AT and 5-15 µg/ml for AB. The correlation coefficient for AT and AB was 0.9992 and 0.9998 respectively. The recovery studies ascertained the accuracy of proposed method and the results were validated as per ICH guidelines. This novel method can be used for the routine quality control of both drugs in combination in tablet dosage form.

INTRODUCTION: Atenolol, 4-[2'-hydroxy-3'-[(1-methyl ethyl) amino] propoxy- benzene acetamide is an odorless white powder, sparingly soluble in water, soluble in ethanol/methanol and practically insoluble in ether is used as an antihypertensive and an antiarrhythmic agent. AT is β_1-selective antagonist. It binds at β_1 adrenergic receptors in the heart and vascular smooth muscle, inhibiting sympathetic stimulation.

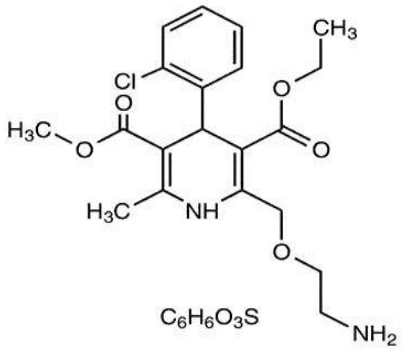
This results in reduction in heart rate, cardiac output, systolic and diastolic blood pressure. AT have an absolute bioavailability of 50% and half life of about 6-7 hours [1]. Amlodipine Besylate, 3-Ethy l-5-methyl (±)-2-[(2-aminoethox) methyl]-4-(2-chlorophenyl)-1, 4-dihydro- 6- methyl- 3, 5-pyridinedi carboxylate, mono benzenesulphonate is a white crystalline powder, soluble in alcohol and practically insoluble in water is used as an antihypertensive and an antianginal agent. AB is a calcium channel blocker [2] which inhibits the influx of extracellular calcium across the myocardial and vascular smooth muscle cell membranes, thus decreases contractile process and hence dilates coronary and systemic arteries. AB has an absolute bioavailability of 64-90% and half life of about 30-50 hours. Fixed dose combination containing AT (50 mg) and AB (5 mg) are available in market as tablets.

AT tablet is official in I.P [3], B.P. [4] and U.S.P. [5]. AB tablet is official in I.P. and E.P. [6]. It is official as Active Pharmaceutical Ingredient in B.P. [7]. Literature survey revealed the availability of several methods for the estimation of AT (non aqueous titration, colorimetry, HPLC, UPLC methods) and AB (colorimetry, HPLC,

HPTLC, LC-MS/MS, electrochemical methods) alone and in combination with other drugs [8-11]. But it was found that there were very few methods reported for the simultaneous estimation of AT and AB from their combination dosage forms [12]. Therefore, a successful attempt was made to develop a new, rapid and selective method for simultaneous estimation of AT and AB from tablet dosage form. The newly developed method was validated as per ICH norms to confirm the reproducibility and wide applicability of the method.



(A) ATENOLOL



(B) AMLODIPINE BESYLATE

FIG. 1: CHEMICAL STRUCTURE OF (A) ATENOLOL (B) AMLODIPINE BESYLATE

MATERIALS AND METHOD:

Instrumentation: High Performance Liquid Chromatograph from Agilent Technologies 1200 series equipped with auto injector, degasser, single head dual plunger pump G1311A (quaternary) for constant flow and constant pressure delivery and UV detector with deuterium lamp linked to software EZ-Chrome Elite for controlling the instrumentation as well as processing the data generated was used. UV-Visible double beam spectrophotometer from Shimadzu 2450 model with spectral slit width of 1.0 nm and automatic wavelength corrections with 10 mm matched quartz cells linked to UV-Probe software was used for analytical wavelength selection. All weighing were done on electronic balance (Model: Sartorious CT-225D). Ultrasonicator (Model: Fast clean 2K911009) and pH meter (Model: Poloman LP-139S) was used for solution preparation and pH determination respectively.

Regents and Chemicals: AT and AB pure drug samples and combination tablets containing 50 mg AT and 5 mg AB manufactured by M.S.N. Laboratories Ltd., Formulations Division, Bollaram, Hyderabad was used. Acetonitrile and Methanol (HPLC grade) from Standard Company, Hyderabad, Triethylamine and Ortho-Phosphoric Acid (AR grade) from Rankem and Milli-Q Purified water (In-house preparation) was used through out the work.

Selection of Analytical Wavelength:

Preparation of mixed stock solution: An accurately weighed 10 mg each of AT and AB working standards were transferred into a 100 ml volumetric flask. 20 ml of methanol was added into it and sonicated for awhile for dissolving the drugs. The flask was made up to 100 ml with methanol so as to get a concentration of 100 µg/ml.

Preparation of mixed standard solution: From the stock solution, 1ml was transferred into a 10 ml volumetric flask and volume was made up to mark with methanol to get a final concentration of 10 µg/ml. The resulting solution containing 10 µg/ml of AT and 10 µg/ml of AB were scanned in UV- Visible spectrophotometer from 400-200 nm to determine the wavelength of maximum absorption of both the drugs in combination. Both drugs in combination showed maximum absorption at 225 nm. The values are shown in **table 1** and the spectra of combination of AT and AB was appended in **Fig. 2**.

TABLE 1: OBSERVATION FROM UV-VISIBLE SPECTRO PHOTOMETER

Sl. No.	Wavelength (nm)	Absorbance of Combination
1	200	0.601
2	210	0.744
3	220	0.702
4	225	0.705
5	230	0.687
6	250	0.229
7	350	0.135
8	400	0.023

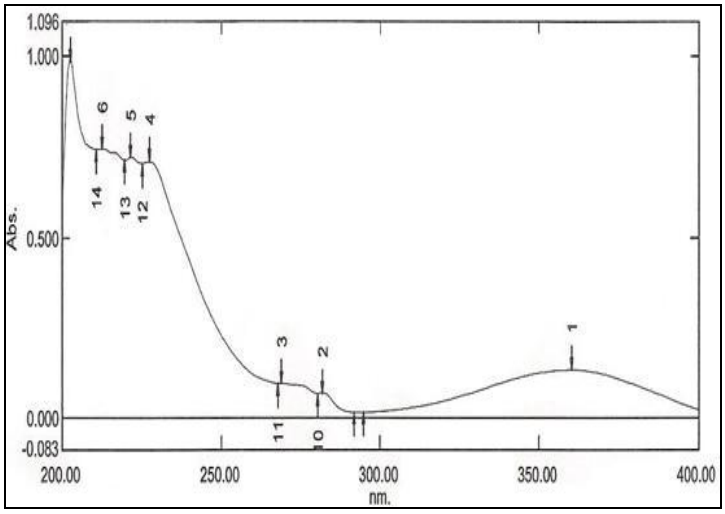


FIG. 2: COMBINED SPECTRA OF ATENOLOL AND AMLODIPINE BESYLATE

Optimized HPLC conditions: Inertsil C_{18} (250 mm x 4.6 mm, 5µm) column maintained at ambient temperature was used as stationary phase. An isocratic mobile phase constituting Triethylamine Buffer (pH adjusted to 3.0 with Ortho-Phosphoric Acid), Acetonitrile and Methanol in ratio 4:3.5:2.5 (v/v/v), at a flow rate of 1.0 ml/min was used. The mobile phase was filtered using 0.45 µm filter paper and degassed for 10 min by sonication. Samples of 10 µl were injected into the HPLC system and the effluents were analyzed at 225 nm. The runtime was 10 min.

Standard preparations:

Standard Stock Solution: Standard stock solution was prepared by transferring 14 mg of AB working standard into 20 ml volumetric flask, added methanol and sonicated for 5 min for dissolving the drug and volume was made up with mobile phase.

Working Standard Solution: Working standard solution was prepared by transferring 10 mg of AT into 100 ml volumetric flask, added methanol and sonicated for 5 min for dissolving the drug. 2 ml of AB stock solution was added and volume was made up with mobile phase.

Sample preparation for Tablet Analysis: Tablet powder equivalent to weight of one tablet was accurately weighed and transferred into 100 ml volumetric flask, 60 ml of mobile phase was added and sonicated for 15 min; volume was made up with mobile phase. Solution was filtered through 0.45µ nylon filter. 5 ml of the filtrate was diluted to 25 ml with mobile phase. 10 µl of the blank, placebo, standard and sample solution were injected separately into the chromatographic system and chromatograms were recorded and the peak areas were measured. A typical chromatogram of AT and AB was appended in **Fig. 3**.

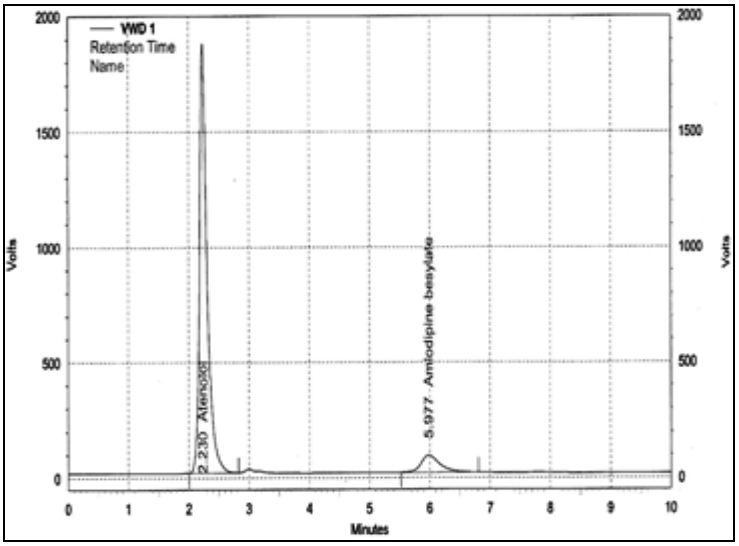


FIG. 3: TYPICAL CHROMATOGRAM OF ATENOLOL AND AMLODIPINE BESYLATE

Method Validation: Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use [13]. The newly developed RP-HPLC method was validated as per International Conference on Harmonization (ICH) guidelines for parameters like system suitability, linearity and range, precision (repeatability), intermediate precision (ruggedness), specificity, accuracy and robustness [14-16].

System suitability testing: A standard solution was prepared using AT and AB working standard as per the test method and was injected six times into the HPLC system. The parameters namely USP plate count, peak asymmetry factor and resolution for the standard solutions were calculated and the values were given in **table 2**.

TABLE 2: SYSTEM SUITABILITY STUDIES

Sl. No.	Parameters	AT[a]	AB[b]
1	USP Plate Count	3527	4441
2	Peak Asymmetry	1.46	1.36
3	Resolution	0.0	9.9

a, b = mean of 6 readings

Specificity (Sensitivity or Selectivity): Specificity was performed to detect the presence of interference peak (blank and placebo peaks) at the retention time of the analyte peak. The interference of placebo was detected by preparing samples by taking the placebo equivalent to about the weight in portion of test preparation as per the test method and were injected into the HPLC system. The interference of blank was detected by injecting mobile phase as per the test method.

Precision: Repeatability (System precision & Method precision): System precision was determined by estimating the %RSD of the peak area for five replicate injections of the standard solution. Method precision was determined by preparing six samples as per the test method representing a single batch. The assay of these samples was determined and the precision of the method was evaluated by computing the %RSD. The values were given in **table 3**.

TABLE 3: PRECISION STUDIES (REPEATABILITY)

Sl. No.	System Precision		Method Precision	
	Standard Area		%Assay	
	AT	AB	AT	AB
1	100.3	99.8	100.0	99.6
2	99.9	99.4	99.9	99.6
3	99.5	99.4	99.2	100.2
4	100.3	99.6	99.9	99.6
5	100.0	99.4	99.7	99.5
6	100.0	99.5	100.1	99.6
Mean	100.0	99.5	99.8	99.7
SD	0.30	0.16	0.32	0.26
%RSD	0.30	0.16	0.32	0.26

SD = Standard Deviation; RSD = Relative Standard Deviation

Precision: Ruggedness (Intermediate precision): The ruggedness of the test method was determined by carrying out precision study in six replicates of assay on a single batch sample in different days by two different analysts, on two different columns and on two different instruments. The difference in the average assay of method precision and intermediate precision for AT and AB is 0.19 and 0.18 respectively which is not more than the limit 2. The values were given in **table 4**.

TABLE 4: PRECISION STUDIES (RUGGEDNESS)

Sl. No.	Analyst 1, Day 1		Analyst 2, Day 2	
	% Assay		% Assay	
	AT	AB	AT	AB
1	100.3	99.8	100.0	99.6
2	99.9	99.4	99.9	99.6
3	99.5	99.4	99.2	100.2
4	100.3	99.6	99.9	99.6
5	100.0	99.4	99.7	99.5
6	100.0	99.5	100.1	99.6
Mean	100.0	99.5	99.8	99.7
SD	0.30	0.16	0.32	0.26
%RSD	0.30	0.16	0.32	0.26

Accuracy (Recovery or Trueness): The accuracy of the test method was determined by preparing recovery samples (spiking placebo with known quantities of AT and AB standard) at the level of 50%, 100% and 150% of targeted concentration. The recovery samples were prepared in triplicate at each level. The samples at different levels were chromatographed and the percentage recovery for the amount added was estimated. The precision of the recovery at each level was determined by computing the %RSD of triplicate recovery results. The values were given in table no. 5.

TABLE 5: RECOVERY STUDIES (ACCURACY)

Spike Level (%)	Added Amt.(ppm)	Recovered [c] Amt. (ppm)	Mean % Recovery	%RSD
AT 50	49.75	50.15	100.80	0.52
100	99.50	99.07	99.57	0.25
150	149.25	149.09	99.90	0.44
AB 50	5.02	5.00	99.67	0.06
100	10.11	10.10	99.87	0.57
150	14.95	14.99	100.23	0.15

c = mean of 3 readings

Linearity: The linearity of detector response for AT and AB was determined by preparing a series of solution of AT and AB working standards over the range of 50% to 150% of targeted concentration. These solutions were injected into the chromatographic system and response area was recorded. The regression equation for AT and AB were found to be y = 20158x+33346 and y = 20124x + 453.44 with correlation coefficient 0.9992 and 0.9998, respectively. A linearity plot for AT and AB was shown in **Fig. 4** and **5**, and the correlation co-efficient was evaluated. The values were given in **table 6**.

TABLE 6: LINEARITY STUDIES

Linearity Level (%)	AT		AB	
	Conc.	Area[d]	Conc.	Area[e]
50	50	1078115	5	99431
75	75	1571158	7.5	153203
100	100	2026472	10	203238
125	125	2573288	12.5	252746
150	150	3030131	15	300303
R^2	y = 20158x + 33346		y = 20124x + 453.44	
m	20158		20124	
c	33346		453.44	
r	0.9992		0.9998	

d, e = mean of 3 readings Conc. = Concentration in ppm; R^2 = Regression Equation; m = Slope; c = Intercept; r = Correlation Coefficient

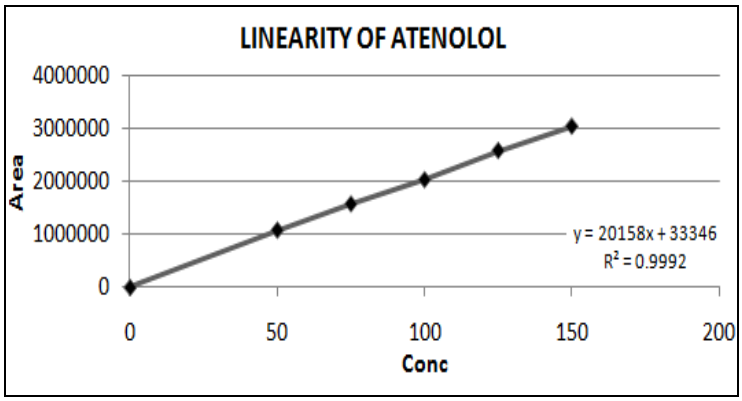


FIG. 4: LINEARITY PLOT FOR ATENOLOL

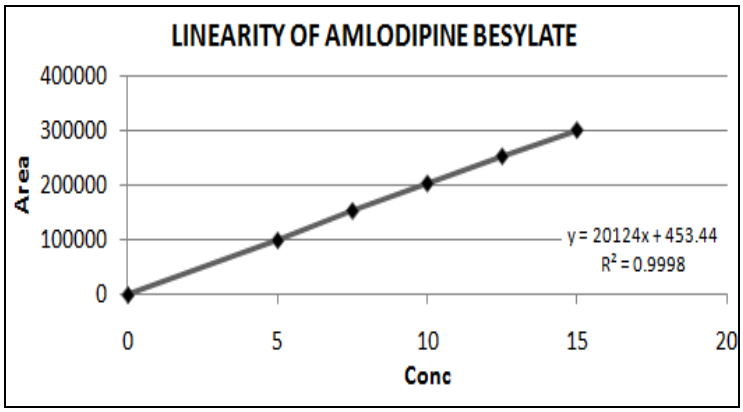


FIG. 5: LINEARITY PLOT FOR AMLODIPINE BESYLATE

Robustness: Effect of variation in flow rate: A study was conducted to determine the effect of variation in the flow rate. Standard solution prepared as per the test method was injected into the HPLC system by keeping flow rates 0.8 ml/min, 1.0 ml/min and 1.2 ml/min and the system suitability parameters were evaluated. The values were given in **table 7**.

TABLE 7: ROBUSTNESS (FLOW RATE) STUDIES

Flow Rate (ml/min)	AT		AB	
	Std Area[f]	Tailing[g]	Std Area[h]	Tailing[i]
0.8	2526823	1.50	255095.2	1.40
%RSD	1.21	0.47	1.29	0.32
1.0	2000287	1.45	197704.4	1.35
%RSD	0.60	1.15	1.35	0.84
1.2	1650522	1.40	161985.6	1.19
%RSD	0.52	0.39	0.92	0.59

f, g, h, i = mean of 5 readings

Robustness: Effect of variation in temperature: A study was conducted to determine the effect of variation in the temperature. Standard solution prepared as per the test method and was injected into the HPLC system at 20°C, ambient and at 30ºC temperature and the system suitability parameters were evaluated. The values were given in **table 8**.

TABLE 8: ROBUSTNESS (TEMPERATURE) STUDIES

Temp. (˚C)	AT		AB	
	Std Area[j]	Tailing[k]	Std Area[l]	Tailing[m]
20	2072960	1.89	194664	1.53
%RSD	0.20	0.24	0.93	0.36
Ambient	2005386	1.47	194000	1.36
%RSD	0.20	0.57	1.25	0.33
30	2204814	1.44	216892	1.10
%RSD	0.14	0.69	0.70	0.76

j, k, l, m = mean of 5 readings; Temp. = Temperature

Robustness: Effect of variation in filter paper: A study was conducted to determine the effect of variation in the filter paper. Standard solution prepared as per the test method was filtered through 0.45 µm membrane nylon filter paper and 0.45 µm PVDF (Poly Vinyl Di Fluoride) filer paper and injected into the HPLC system. The percentage difference in assay in test solution with both the filter papers was evaluated. The values were given in **table 9**.

TABLE 9: ROBUSTNESS (FILTER PAPER) STUDIES

Sl. No.	Percentage Assay		
	Nylon[n]	PVDF[o]	Diff. (%)[p]
AT	100.10	99.63	0.47
AB	99.63	99	0.70

n, o, p = mean of 3 readings; Diff. = Difference

RESULTS AND DISCUSSION: A new RP-HPLC method was developed for the simultaneous estimation of AT and AB in tablet formulation and validated as per ICH norms for the following parameters: system suitability, linearity and range, precision (repeatability), intermediate precision (ruggedness), specificity, accuracy and robustness. The observations and results obtained for each of the parameters lies well within the acceptance criteria. So the developed method was simple, specific, linear, precise, accurate, robust and rugged and could be extensively used for the simultaneous estimation of AT and AB in tablet dosage form.

System suitability parameters proved that the proposed method suits for the simultaneous estimation of AT and AB. After various trials performed, chromatogram for AT and AB was found satisfactory on Inertsil C_{18}, 5µm, 250 mm x 4.6 mm, using mobile phase composition of buffer: acetonitrile: methanol (4: 3.5: 2.5). Drug peak was found to be symmetrical as observed from asymmetry factor of 1.46 for AT and 1.36 for AB. Resolution of the proposed method was found to be satisfactory.

Sensitivity of the method was good and also linearity was observed over a wide concentration range of 50-150 µg/ml for AT and 5-15 µg/ml for AB. Accuracy of the method was determined by recovery with spiked concentration of pure drug at five levels for AT and AB. Recovery of drug was well within the acceptance limits of 97-103%. Method was robust and rugged as observed from insignificant variation in the results of analysis on changes in flow rate, temperature, filter and analysis being performed by different analysts, in different days on different systems using different columns respectively.

CONCLUSION: From the results obtained, it was observed that the developed method was proven to be specific, precise, linear, accurate, rugged and robust and is suitable for its intended purpose. So the work performed gives documented evidence, that the analytical method for the simultaneous estimation of AT and AB by RP-HPLC in tablet dosage forms will consistently analyze these drugs quantitatively and can be used for routine analysis in quality control and R&D laboratory.

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