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A New RP-HPLC Method Development and Validation for Simultaneous Estimation of Atenolol and Alprazolam in Tablets

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ABSTRACT

The present work describes a new, simple, precise, and accurate RP-HPLC method for simultaneous estimation of atenolol and alprazolam in bulk and formulation. Chromatographic separation of the drugs was achieved on a BDS (250mm 4.6mm,) 5µm using acetonitrile: buffer (0.01KH_2PO_4 pH 3) in the ratio of 60:40 v/v as a mobile phase. The flow rate was 1.0 mL/min and the detection wavelength was 210 nm. The two drugs were satisfactorily resolved with retention time values 2.640 min and 3.990 min for atenolol and alprazolam, respectively. The method was validated in terms of accuracy, precision, linearity, and limit of detection, limit of quantitation, robustness, and ruggedness as per ICH guidelines. Linearity was found to be in the range of 50-300 mcg/ml for atenolol and 0.5-3.0 mcg/ml for alprazolam with the significantly high value of a coefficient of determination (r^2 = 0.9995 for atenolol and 0.9991 for alprazolam). The accuracy and reliability of the method were assessed by evaluation of precision (intra-day and inter-day precision % RSD was less than 2% for both atenolol and alprazolam), accuracy (99.8% for atenolol and 99.84% for alprazolam), and specificity, in accordance with ICH guidelines. The LOD and LOQ were found to be 0.10µg/ml and 0.31µg/ml for atenolol and 0.60µg/ml and 0.81µg/ml for alprazolam respectively. The proposed method can be used for the estimation of these drugs in combined dosage forms.

INTRODUCTION

Atenolol is chemically 2-[4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl]acetamide. Atenolol, a competitive beta (1)-selective adrenergic antagonist, has the lowest lipid solubility of this drug class. Although it is similar to metoprolol, atenolol differs from pindolol and propranolol in that it does not have intrinsic sympathomimetic properties or membrane-stabilizing activity. Atenolol[1] has used alone or with chlorthalidone in the management of hypertension and edema. Atenolol competes with sympathomimetic neurotransmitters such as catecholamines for binding at beta (1)-adrenergic receptors in the heart and vascular smooth muscle, inhibiting sympathetic stimulation. This results in a reduction in resting heart rate, cardiac output, systolic and diastolic blood pressure, and reflex orthostatic hypotension. Higher doses of atenolol also competitively block beta (2)-adrenergic responses in the bronchial and vascular smooth muscles.

Alprazolam[2]is chemically 8-Chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine. Alprazolam is the Triazolobenzodiazepine compound with antianxiety and sedative-hypnotic actions that is efficacious in the treatment of panic disorders, with or without agoraphobia, and in generalized anxiety disorders. Alprazolam, a benzodiazepine, is used to treat panic disorder and anxiety disorder. Unlike chlordiazepoxide, clorazepate, and prazepam, alprazolam has a shorter half-life and metabolites with minimal activity. Like other triazolo benzodiazepines such as triazolam, alprazolam may have significant drug interactions involving the hepatic cytochrome P-450 3A4 isoenzyme[3]. Clinically, all benzodiazepines cause a dose-related central nervous system depressant activity varying from mild impairment of task performance to hypnosis. Unlike other benzodiazepines, alprazolam may also have some antidepressant activity, although clinical evidence of this is lacking.

Various analytical methods reported estimation of atenolol and alprazolam in formulations. These are mainly spectrophotometric, colorimetric and chromatographic methods in individual or in combination with other drugs. Hence in the present work, a simple precise and accurate, RP-HPLC method was developed for the estimation of atenolol and alprazolam in combined dosage forms.

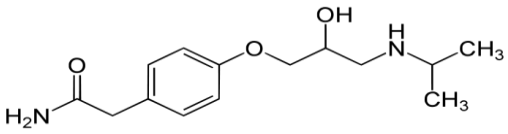

Fig 1: Structure of Atenolol

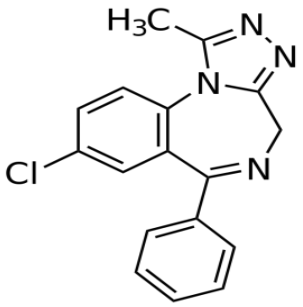

Fig 2: Structure of Alprazolam

EXPERIMENTAL

Materials, chemicals, and reagents

Atenolol and alprazolam standard (Lot No. 042281) (Lot No. 042124) was provided by Hetero Pharma Ltd. (Hyderabad).Atenolol and alprazolam tablets (TENORMIN) containing 2 mg alprazolam and 25 mg atenolol were obtained from local market. Analytical grade potassium dihydrogen phosphate, ammonium acetate were purchased from S.S. fine chemicals, Hyderabad. HPLC grade acetonitrile and water were obtained from Merck, Mumbai.

Instrumentation

HPLC instrument used was of Waters HPLC 2965 System with Auto-Injector and PDA detector with the software Empower 2. UV-VIS spectrophotometer, PG Instruments, T60 with the special bandwidth of 2mm and 10mm and matched quartz was used for measuring absorbance for atenolol and alprazolam solutions.

Mobile phase preparation

The mobile phase consisted of 0.01M potassium dihydrogen phosphate buffer (pH3.0) and acetonitrile in the ratio of 60:40. 1.36 g of potassium dihydrogen phosphate was weighed and dissolved in 1000 ml of HPLC grade water. 600 ml from above solution was added to 400 ml of acetonitrile. The mobile phase was subjected to ultrasonication and vacuum filtration.

Diluent preparation

Mobile phase was used as diluent.

Wavelength detection

Detection of absorbance's of atenolol and alprazolam was performed at 210 nm.

Chromatographic conditions

Chromatographic analysis was performed on an Inertsil ODS C18, 250x 4.6mm,5µm. column. The mobile consisted of 0.01M KH_2PO_4 (pH3.0) buffer and acetonitrile in the ratio of 55:45. The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 20 µl. Detection was performed at 210 nm.

METHOD DEVELOPMENT

Standard preparation:

Accurately weighed and transferred 2mg of atenolol and 20mg of alprazolam into 100ml and 10ml volumetric flasks separately. 3/4ml of diluent was added and sonicated for 30 minutes and the final volume was made up with the diluent. .From the above stock solutions, 1 ml was pipetted out into a 10ml volumetric flask and the final volume was make up to 10 ml with the diluent.

Test preparation

Five tablets were weighed and the average weight of each tablet was measured, then the weight equivalent to five tablets was transferred into a 50 ml volumetric flask,30ml of diluent was added

and sonicated for 25 min, further, the volume made up with diluent and filtered. From the filtered solution, 0.4ml was pipetted out into a 10 ml volumetric flask and made up to volume 10ml with diluent.

Calculation

The amount of atenolol and alprazolam present in the formulation by using the formula given below, and results are shown in above table:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to sample preparation

WS: Weight of standard in mg

WT: Weight of sample in mg

DT: Dilution of assay preparation

AW: Average weight of sample

LC: Label claim

P: Potency of pure drug

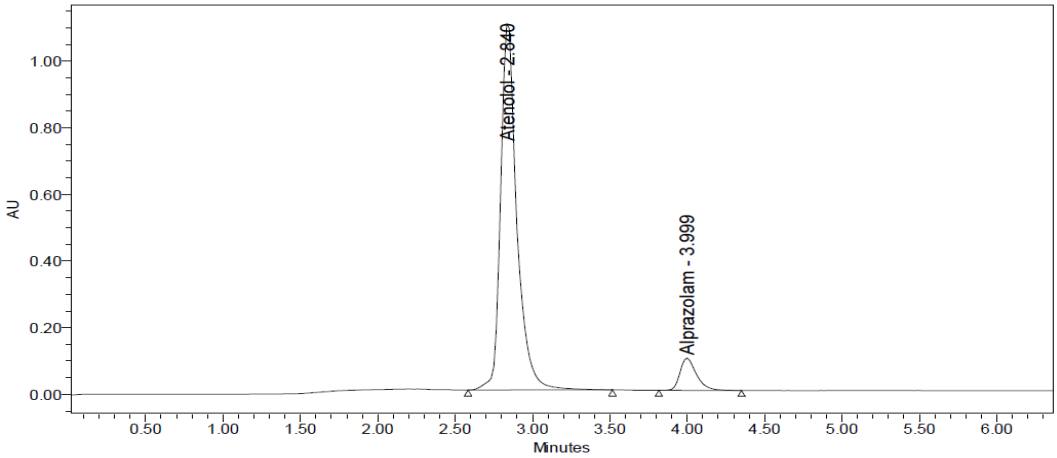

Fig 3: Chromatogram of standard preparation of atenolol and alprazolam

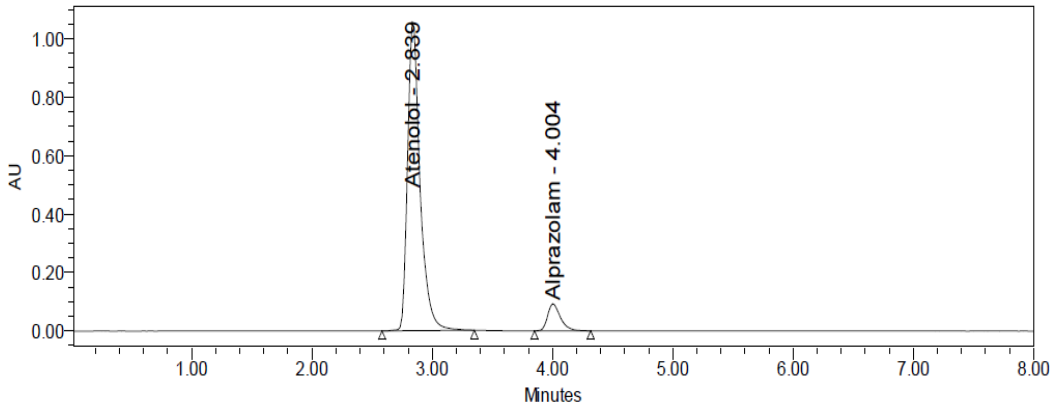

Fig 4: Chromatogram of sample preparation of atenolol and alprazolam

Table: 1 Assay results of atenolol and alprazolam

Sr. No.	Atenolol % Assay	Alprazolam % Assay
1	99.72	99.70
2	99.40	99.74
3	100.68	101.39
4	98.13	99.29
5	100.06	100.12
6	100.15	100.20
AVG	99.69	100.07
STDEV	0.8783	0.72
% RSD	0.88	0.72

METHOD VALIDATION

System suitability study

A system suitability test for the chromatographic system was performed before each validation experiment. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate, and % RSD of peak area were determined for same. Only after the system suitability results were in acceptance criteria the experiments were preceded further.

Table: 2 Results for system suitability of atenolol

Injection	Retention time (min)	Peak area	Theoretical plates (TP)	Tailing factor (TF)
1	2.839	8073421	3412	1.321
2	2.840	8195114	3415	1.443
3	2.841	8053316	3401	1.386
4	2.840	7967499	3301	1.414
5	2.838	8101712	3983	1.365
6	2.841	8124031	3701	1.414
Mean	2.839	8084859	-	-
SD	0.001	7.598	-	-
% RSD	0.010	1.71	-	-

Table: 3 Results for system suitability of alprazolam

Injection	Retention time (min)	Peak area	Theoretical plates	Tailing factor	Resolution
1	3.999	726851	2930	1.603	3.617
2	4.010	721045	2801	1.621	3.612
3	4.100	710625	2569	1.617	3.512
4	3.998	721641	2864	1.545	3.612
5	4.011	710817	2797	1.447	3.712
6	4.001	720125	3021	1.701	3.512
Mean	4.019	718517	-	-	-
SD	0.039	64.973	-	-	-
% RSD	0.36	0.51	-	-	-

Acceptance criteria

1. The % RSD for the retention times of atenolol and alprazolam peaks from 6 replicate injections of each standard solution should be not more than 2.0 %

2. The % RSD for the peak area responses of atenolol and alprazolam peaks from 6 replicate injections of each standard solution should be not more than 2.0%.
3. The number of theoretical plates (N) for the atenolol and alprazolam peaks is not less than 2000.
4. The tailing factor (T) for the atenolol and alprazolam peak is not more than 2.0.

Conclusion:

The theoretical plates should not be less than 2000, asymmetry should be less than 2.0 and % RSD should be less than 2.0. As the data suggest the system suitability was within the criteria in each validation experiment for atenolol and alprazolam.

Specificity

In an assay, demonstration of specificity requires that it can be shown that procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay results is unaffected by the presence of these extraneous materials.

Conclusion:

There should be no interference of the diluents, placebo at the retention time of drug substances.

Linearity:

Six Linear concentrations of atenolol (50-300µg/ml) and alprazolam (0.5 µg/ml to 3 µg/ml) are prepared and injected. Regression equation of the atenolol and alprazolam were found to be, y = 40922x + 1266, y = 34636x + 6264 and regression co-efficient was 0.999.

Linearity solutions are prepared such that 0.25ml, 0.5ml, 0.75ml, 1ml, 1.25ml, 1.5ml from the Stock solutions of atenolol and alprazolam are taken in to 6 different volumetric flasks and diluted to 10ml with diluents to get 50, 100, 150, 200, 250, 300 µg/ml of atenolol and 0.5, 1, 1.5, 2, 2.5, 3 µg/ml of alprazolam.

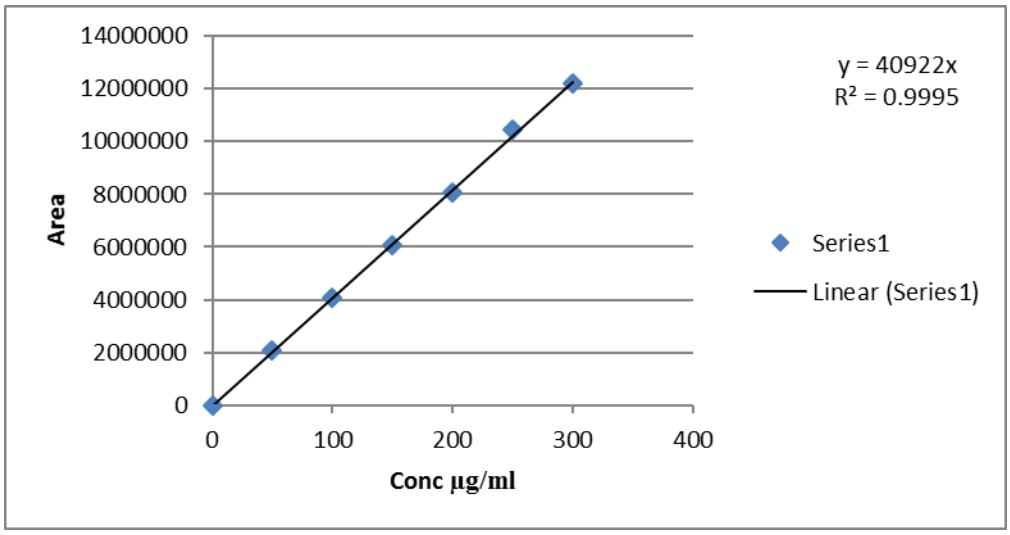

Fig 5: Calibration curve of atenolol

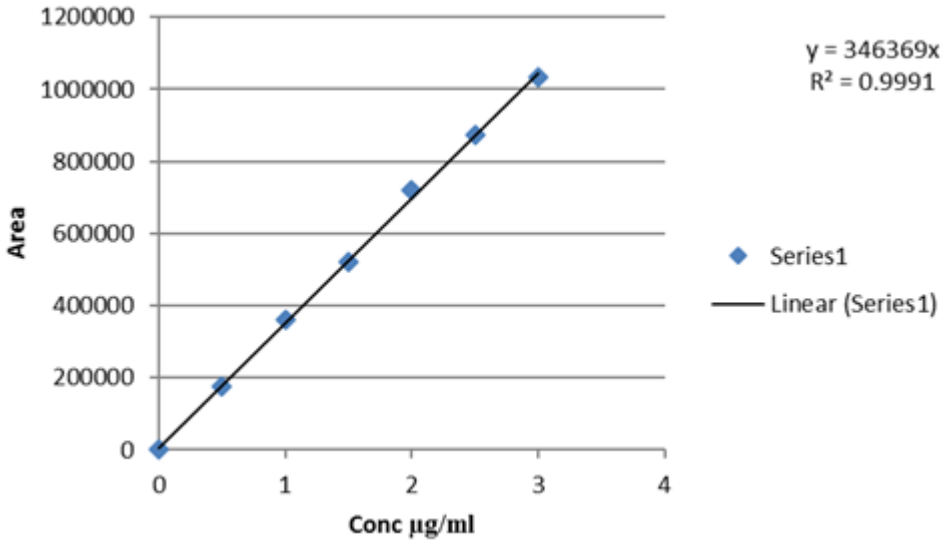

Fig 6: Calibration curve of alprazolam

Conclusion:

The coefficient of determination for linear curve obtained between concentration vs. area for standard preparations of atenolol and alprazolam is 0.9991. The relationship between the concentration of atenolol and alprazolam and area of atenolol and alprazolam is linear in the range examined since all points lie in a straight line and the coefficient of determination is well within limits.

Limit of detection and Limit of quantitation study:

LOD is the lowest amount of the drug content which can be detected by the proposed method while LOQ is the lowest amount which can be quantified by the method. The guideline suggests minimum signal to noise ratio (S/N) more than 3.3 for LOD and more than 10 for LOQ. On the basis of linearity data theoretically, it can be also calculated by the given formula.

$LOD = 3.3\ \sigma/S$ $LOQ = 10\ \sigma/S$

Where σ = residual standard deviation of regression line and S=slope of regression line

Conclusion;

LOD for atenolol and alprazolam were found to be 0.10 µg/ml and 0.60 µg/ml respectively.

LOQ for atenolol and alprazolam were found to be 0.31 µg/ml and 0.81 µg/ml respectively.

Precision:

Intraday precision (Repeatability):

The precision of test method was evaluated by analyzing six samples.

Acceptance Criteria:

Relative standard deviation; NMT 2 %

Intermediate precision:

It expresses within laboratory variations; different days, different analysts, different equipment, etc. Conduct analyst to analyst variability by two different analysts using same are different HPLC system qualified during system to system variability and using the same or different HPLC column qualified during column to column variability, by injecting six samples prepared samples.

Table:4 Repeatability results for atenolol and alprazolam.

Sr. No.	Atenolol	Alprazolam
1	8075868	716817
2	8049314	717122
3	8153306	728925
4	7946489	713841
5	8102612	719817
6	8110331	720424
Mean	8072987	719491
Std. Dev.	71125.0	5189.6
%RSD	0.88	0.72

Acceptance criteria:

Relative standard deviation is NMT 2

Conclusion:

Intraday precision: Inter-day precision was performed with 24 hrs time lag and the %RSD obtained for atenolol and alprazolam were 0.88 and 0.7

Table: 5 Inter-day precision results for atenolol and alprazolam

Sr. No.	Atenolol	Alprazolam
1	7985479	720637
2	8102945	728381
3	8008503	715439
4	8064897	725787
5	8049164	721034
6	8115412	726318
Mean	8054400	722933
Std. Dev.	51133.8	4781.7
% RSD	0.63	0.66

Fig-4.6.7 Chromatogram of inter-day precision of atenolol and alprazolam

Conclusion:

Inter-day precision was performed with 24 hrs time lag and the %RSD obtained for atenolol and alprazolam were 0.63 and 0.66

Accuracy:

The accuracy of the method was determined by recovery studies. To the formulation (pre-analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for a drug is shown in a table. To check the accuracy

of the method, recovery studies were carried out by an addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100%, 150%.

Table: 7 Accuracy results of atenolol and alprazolam

Sample	Amount added (µg/ml)	Amount recovered (µg/ml)	Recovery (%)	% RSD
Atenolol - 20mg	10	9.85	99.85	1.43
	20	19.95	99.77	0.56
	30	29.94	99.83	0.74
Alprazolam-2mg	10	9.98	99.79	1.08
	20	20.06	100.3	0.67
	30	29.86	99.52	0.67

Conclusion:

The % recovery of atenolol and alprazolam should lie between 98% and 102%.

Robustness:

To demonstrate the robustness of the method, prepared solution as per test method and injected at different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision. Small deliberate changes in the method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH guidelines.

Table: 8 Robustness data of atenolol and alprazolam

Sr. No.	Robustness condition	Atenolol %RSD	Alprazolam %RSD
1	0.8 ml	0.9	1.2
2	1.2 ml	0.1	0.5
3	Mobile phase 55:45	0.2	0.9
4	Mobile phase 70:30	0.4	0.4
5	Temperature 25^0C	0.3	0.3
6	Temperature 30^0C	0.8	0.7

Conclusion:

The result showed that during all variance conditions, assay value of the test preparation solution was not affected and it was accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

RESULTS AND DISCUSSION

The stated method represents simultaneous method development and method validation of atenolol and alprazolam in bulk and pharmaceutical dosage forms. Various analytical methods reported estimation of atenolol and alprazolam in formulations. These are mainly spectrophotometric, colorimetric and chromatographic methods in individual or in combination with other drugs. Only one chromatographic[4] method reported the estimation of atenolol and alprazolam in bulk and pharmaceutical formulations.

The present method was aimed to develop the novel chromatographic method for estimation of atenolol and alprazolam. Chromatographic conditions were optimized to achieve separation of drug components with the use of BDS column, mobile phase buffer (pH3.0) and acetonitrile in the ratio of 55:45.The method was more economic as less consumption of organic solvents reported which separates and eluted the peak within 5 minutes only. The existing[5] method reported pH of mobile was 9.0. which was more basic effects the performance of column and system suitability studies were not accepted limits which made the method lacking sensitivity. The present method more suitable for daily laboratory analysis of quality control laboratories.

Chromatographic separation of atenolol and alprazolam occur at the retention time of 2.8 and 3.9 min respectively. There was no interference[6] of placebo and impurities. Specificity, linearity, precision studies indicate the sensitivity and suitability of the represented method.

SUMMARY AND CONCLUSION

A simple, accurate, precise method was developed and validated for the simultaneous estimation of the atenolol and alprazolam in tablet dosage form. In this method development was done using BDS column (250mm 4.6mm, 5µm) with Mobile phase containing buffer and acetonitrile[7] in the ratio of 45:55 was pumped through the column at a flow rate of 1ml/min. The buffer used in this

method was 0.01N KH_2PO_4 at pH 3. The temperature was maintained at 30°C. Optimized wavelength for atenolol and alprazolam was 210nm[9]. The retention time of atenolol and alprazolam were found to be 2.8min and 4.0min. %RSD of the atenolol and alprazolam were and found to be 0.88 and 0.72 respectively[8]. % Recovery was Obtained as 99.81% and 99.87% for atenolol[10] and alprazolam respectively. LOD, LOQ values are obtained from regression equations of atenolol and alprazolam were 0.10ppm, 0.31ppm and 0.60ppm, 1.81ppm respectively[11]. Regression equation of atenolol is y = 40922x, and of alprazolam is y = 34636x

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