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Graduation Project Report On

Optimization of a micro-high-performance liquid chromatography method for determination of Alprazolam benzoate (APB) in their standard powder and in dosage pharmaceuticals

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By:

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آية الكرسي سورة البقرة آية ٢٥٥

Certification of the Supervisor

I certify that this project entitled " Optimization of a micro-high-performance liquid Chromatography method for determination of Alprazolam benzoate in their Standard powder and in dosage pharmaceuticals " was prepared by the fifth-year Student under my supervision at the College of Pharmacy/ University of Basra in Partial Fulfillment of the graduation requirements for the Bachelor Degree in Pharmacy.

Assist. Lec. Hussein Nassar

Hussein Hassan

Ali khamas

Dedication

I dedicate this work to God,My creator, my strong pillar, my source of inspiration and wisdom.To Mom & dad,

Thank you for raising me to believe in God, myself and in my dreams.

To my family members For teaching me to believe that everything is possible.

To my best friends For making the world a better place, just by being in it…

Acknowledgment

First of all, I thank "Allah" almighty for granting me the will and strength to Accomplish this project, and I pray that his blessings upon me may continue throughout My life, without "Allah", I would not have had the wisdom or the physical ability to do

So.

Deep thanks to Doctors of Pharmaceutical Chemistry Department , Dean of the

College of Pharmacy, University of Basra, for his support to accomplish this project.Deep thanks to Dr.Hussein Nassar, the associate dean for scientific affairs.I am indeed internally thankful to Dr.Raheem, Head of the Department of Pharmaceutical chemistry, for her kindness and encouragement.I would like to thank my supervisor Dr.Hussein Hassan, for her continuous guidance, Generous advice, encouragement and wise supervision.My great appreciation and thanks to Dr.Ali Khamas for his kind effort and help.Finally, thanks to all of the teaching staff of the Department of pharmaceutical Chemistry.

List of Abbreviations

HPLC …….. High performance liquid concentration

Alp…….Alprazolam

VH ……..Vitrous humor

Dad ……. Diodie array detector

CV ……Coffecient of variation

TLC…… Thin layer chromatography

Spe …….. Soild phse extraction

Lle …….. liquid –liquid extraction

PMI…… Postmortem interval

RSD…….Relative standard deviation

LOD……. Limit of detection

LOQ…… limit of quantitation

HIGHLIGHTS

• A new method of estimating Alprazolam benzoate (APB) in pharmaceuticals.

• Use of HPLC-UV technology for LC100 in the estimation of Alprazolam benzoate(APB).

• Study the structural synthesis of Alprazolam benzoate (APB) in the neutral, acidic and base.

• Studying the relative stability of Alprazolam benzoate during the experimental estimation process.

• Perform different applications for the purpose of validating the chromatographic method in the estimation of Alprazolam benzoate (APB).

Abstract

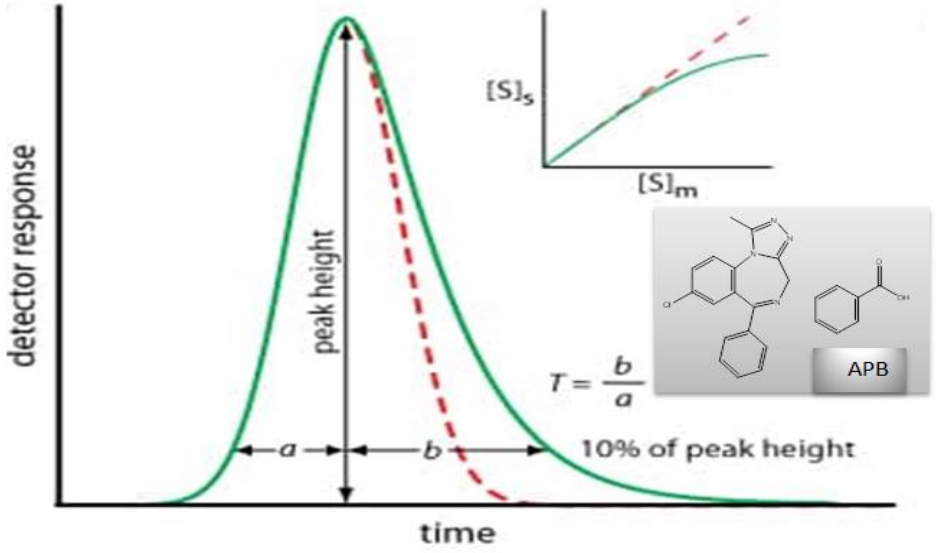
Context: In this manuscript, a high-performance liquid chromatography (HPLC) method for the determination of Alprazolam benzoate (APB) in pharmaceuticals was described and developed. Methods: The reversed-phase HPLC (RP-HPLC) method was developed and the results were obtained to determine the form of (APB). Chromatographic analysis was performed in HPLC-ultraviolet (HPLC-UV) system with Ion Pac column; Arcus EP-C18; 5 µm, 4.6 mm×250 mm, with acetonitrile: triethylamine 30:70 (v/v)+0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 as mobile phase, at a flow rate of 1.0 ml/min. UV detection in the HPLC system was performed at 310 nm. Results: The method was validated for accuracy, precision, specificity, linearity, and sensitivity. The retention time for the (APB) was ~1.10 min. Calibration plots were linear over the concentration ranges 1–5 µg/L for the Alprazolam benzoate. The limit of detection (LOD) was 0.0143 µg/ml and the limit of quantitation (LOQ) was 0.0316 µg/ml.

The accuracy of the proposed method was determined by recovery studies and found to be 100%. Conclusion: Commercial tablet formulation was successfully analyzed using the developed HPLC-UV method that has been validated; accuracy,

precision, and specificity were found to be within acceptable limits. Moreover, results obtained by the suggested methods showed no significant difference between the results obtained from the recommended method.

Keywords: Detection limit, (APB) drug, micro-high-performance liquid chromatography, quantification limit, statistical analysis

Graphical Abstract



INTRODUCTION

Alprazolam benzoate (APB) is used as an antiprotozoal, chemical name AUPIC:

8-chloro-1-methyl-6-phenyl-4H-benzo[f][1],($C_{17}H_{13}CIN_4$,Mol.Wt.308.765), (APB) is a white crystalline powder or light yellow odorless, almost soluble in dichloromethane, chloroform, and soluble acetone in ethanol, almost insoluble in water. Its melting point is 99–102, is a benzoic acid derivative used as antiamebic, antiprotozoal, and antibacterial [Figure 1], [2].

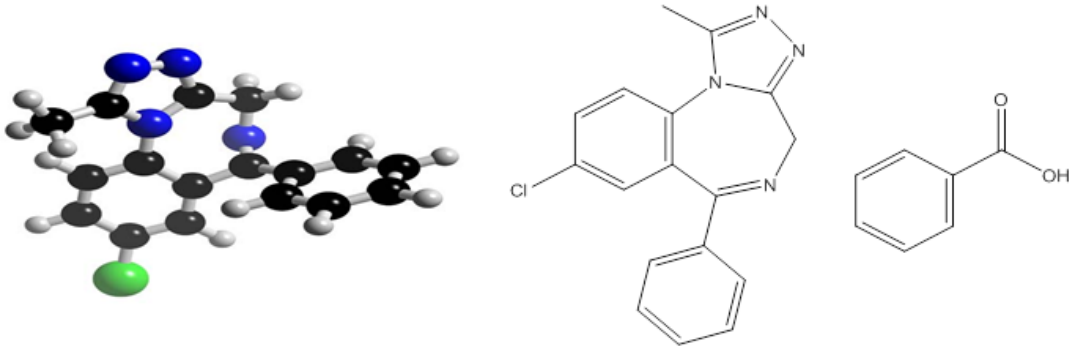


Figure 1: Chemical Stracture of Alprazolam benzoate (APB)

The medical properties of APB are antimicrobial and antimicrobial agents. Nitroimidazole derivatives are also used in the treatment of anaerobic bacterial infections. The drug is converted into anaerobic bacteria by the enzyme pyroxene and verdoxine oxidase. The nitro group in APB is chemically reduced by ferredoxin metabolism or by the associated ferredoxin group. Therefore, the new product is responsible for destroying the structure of the DNA spiral chains, thereby inhibiting the synthesis of DNA in microbial and bacterial organisms [3-5].

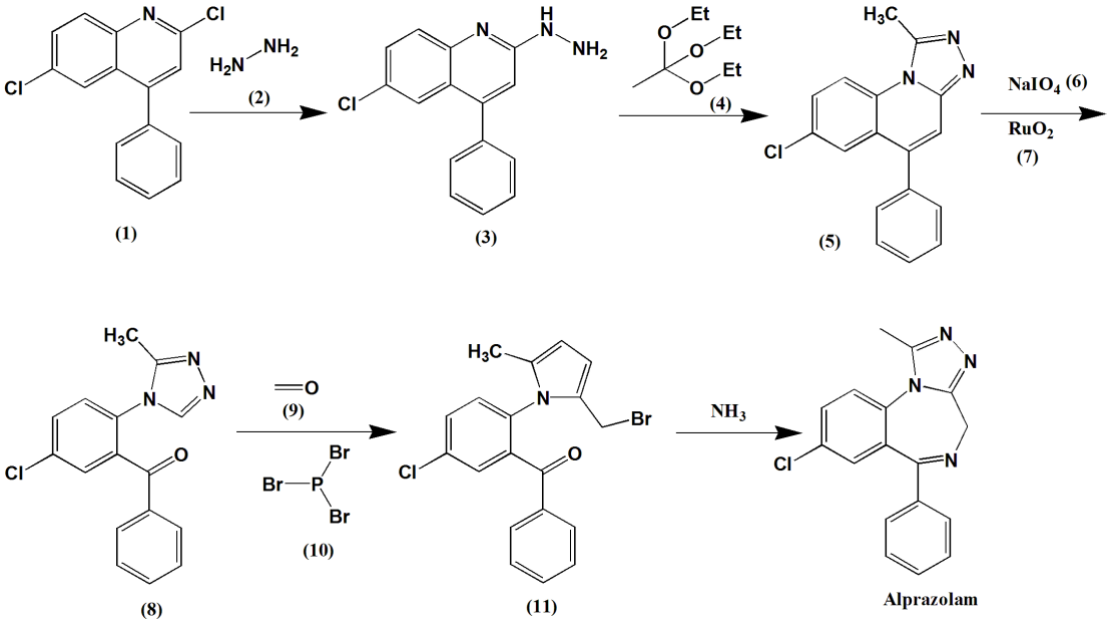
A number of different analytical methods can be used to test and identify APB in pharmaceuticals, especially the high-performance liquid chromatography method, which is considered to be a modern and advanced method. Quantification of APB is measured in pharmaceuticals using high-performance liquid chromatography (HPLC)[6,7].

In this study, reversed-phase HPLC (RP-HPLC) was developed using an ultraviolet detector, a simple, fast, and sensitive method for the quantitative determination of APB in pharmaceuticals. The stability of samples was determined in different laboratory conditions. It is very important to develop an appropriate analytical method to estimate the content of APB in its pharmaceutical forms. In the HPLC method, an eluent solution consisting of a mixture of solvents such as acetonitrile, methanol, and potassium hydrogen phosphate is used. A chromatographic separation column (Ion Pac Arcus EP-C18; 5 μm, 4.5 mm × 250 mm) is selected with a qualitative and quantitative estimate of this type of pharmaceutical and appropriate separation conditions are applied. This method was validated in accordance with the Food and Drug Administration Guidance Document, entitled “Dynamic Verification Method” (May 2001).[8,9].

The RP-HPLC method was also validated in accordance with the International Council for chemical Harmonisation (IHC) guidelines.

Alprazolam Synthesis

APB is produced by imidazole synthesis or ethylenediamine and acetic acid, followed by treatment with lime, then nickel as in the steps of the following equation (Schame 1), [10].



Schame 1: Synthesis of Alprazolam

The objective of the study

The objective of the study was to develop and verify the RP-HPLC method with an ultraviolet (UV) detector for the quantitative determination of APB in pharmaceuticals.

EXPERIMENTAL

Instrumentation

LC-100 series S-HPLC features fully automatic digital computer control. Its electronic circuit design, internal mechanical structure design, processing technology, functions of cinematography workstation, and the technical criteria make it leading instruments with excellent stability and reliability. The LC100-type HPLC-UV consists of a double-beam optical spectrometer (Angstrom Advanced

Inc., USA), model UV-100 PC with 1 cm path length quartz cell is used and it is connected to IBM compatible computer. The software was UVPC personal spectroscopy software version Matlab, R2003b was used for the proposed chemometric methods, the partial least squares (PLS) were performed with PLS_Toolbox for use with Matlab R2003b, VP pumps, and variable wavelength programmable UV detector. Peak areas were integrated using an Angstrom Advanced Inc. LC solution software program. The chromatographic separation and quantification were performed on Ion Pac column; Arcus EP-C18 (250 mm × 4.6 mm; particle size 5 μm) analytical column maintained at room temperature. The mobile phase, drug standard solutions, and tablet sample solutions were filtered through a millipore membrane filter before injection into the HPLC system.[11-14]

CHEMICALS AND REAGENTS

Pure Standard

APB standard with claimed purity of 99.8%, as certified by manufacturer PubCem Drug Industries, USA, for medical devices and pharmaceuticals, Cas number: 28981-97-7, EINECS: 249-349-2, BRN: 1223125, MDL number: MFCD00078881.

Market Sample

Alprazolam LPH- Serie® tablets batch No. 486346- labeled to contain 0,25 mg APB per tablet were manufactured by labormed for Pharmaceuticals and Medical Appliances LPH- Serie®.

Configure the Samples for Measurement

• HPLC grade (Sigma-Aldrich® Chemie GmbH, Germany) solutions

• Stock standard solutions of APB were prepared in acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 to prepare concentration of 1 mg/ml from APB.[15,16]

• Working standard solutions of APB was prepared in acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 to prepare the concentration of 1.0, 2.0, 3.0, 4.0, and 5.0 μg/ml.

Sample Updating

To perform model updating, the optimized PLS calibration set was augmented with different samples of Alprazolam LPH- Serie® tablets containing known amounts from standard APB, were manufactured by Labormed. One known concentration to three unknown concentrations of samples containing different concentrations of each was added purpose for done the initial calibration and the predictive ability of the updated sample was checked using external validation samples, then calculate the perform sample updating for each component using the developed method RP-HPLC with three concentrations of the added updating samples.[17-20]

PROCEDURE

Standard Drug Solution

The mobile phase was used as solvent for the preparation of standard solutions. Standard stock solution of APB (250 µg/mL) was prepared by dissolving an accurately weighed amount of APB (25 mg) in 50 mL of mobile phase in 100 mL volumetric flask. The flask was then made up to the mark with mobile phase. The stock solution was diluted aptly with mobile phase to prepare the working standard solutions of APB (1, 2, 3, 4, and 5 µg/mL).

RESULTS

The Calibration Curve

Calibration curves of the proposed method were prepared over concentration range of 1–5 µg/ml for APB. Solution wasprepared in triplicate and 20 µl of each solution was injected onto the column. The peaks were determined at 310 nm, (Table 1).

The calibration curve of APB was constructed by plotting the peak area versus concentration.

Table 1: shows the values of the basic parameters obtained using the reverse-phase chromatography system (RP-HPLC).

mobile phase	acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5
Run time	10 min
Column temperature	25 °C
Detection wavelength	310 nm
Flow rate	1.0 ml/minute
Injection volume	20 µL

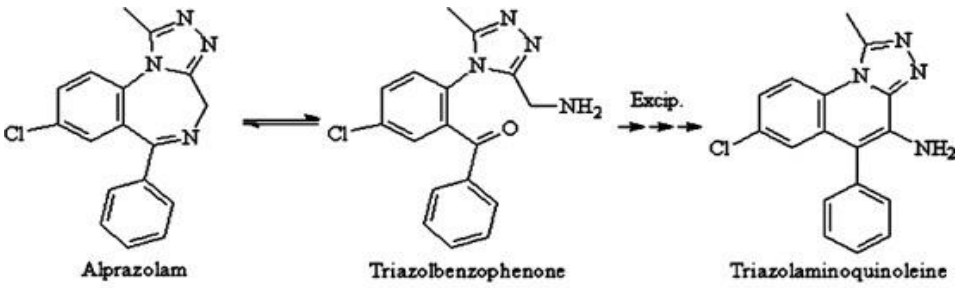
Stress Degradation Studies

Stress degradation studies were carried out using different ICH prescribed stress conditions such as acidic, basic, oxidative, thermal, and photolytic stresses.[21-23]

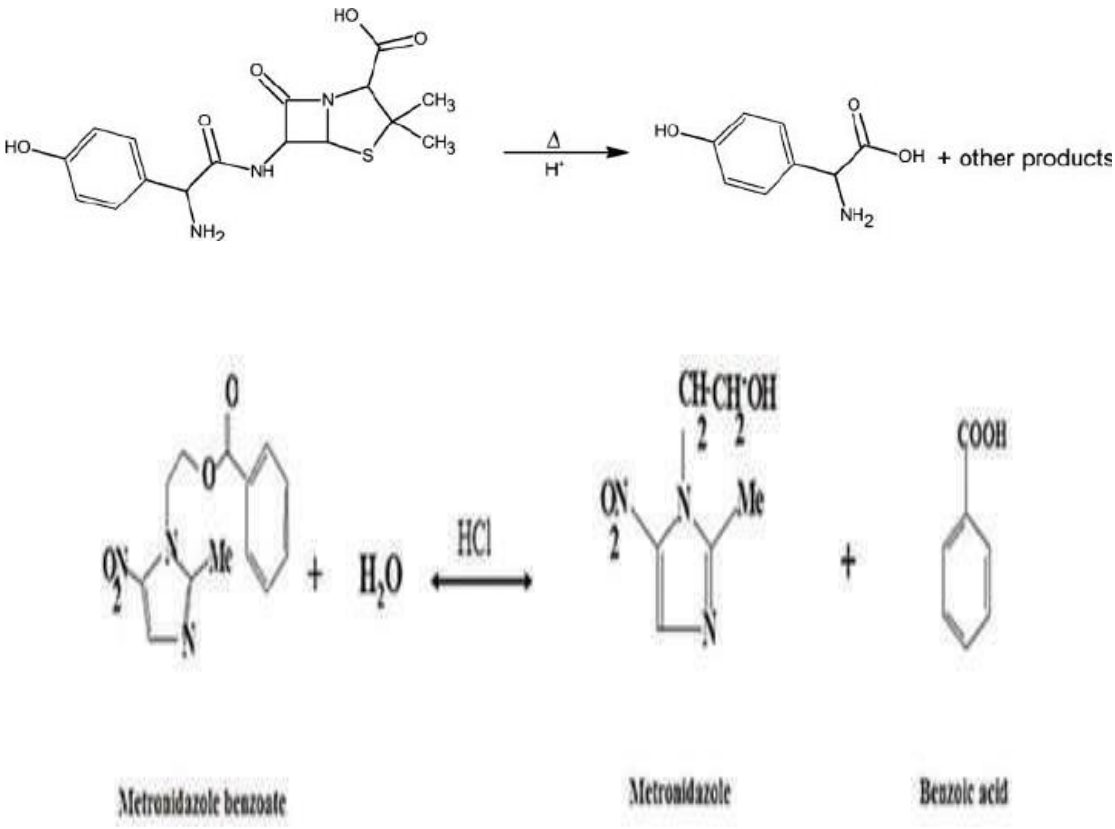
Acid Degradation

About 60 mg from tablet powder of APB was taken in 100 ml volumetric flask. 5 ml of 0.1 N HCl was added to the flask and kept at 70–80°C reflux condition for 2–3 h.

After completion of the stress, the solution was neutralized using 0.1 N NaOH and completed up to the mark with mobile phase. Hydrolysis of APB may be hydrochloric acid.



One such reaction is hydrolysis, “splitting with water.” Hydrolysis of esters is stimulated by any acid or base (Schame 2 and Figure 2).



Schame 2: Structure of the APB benzoate

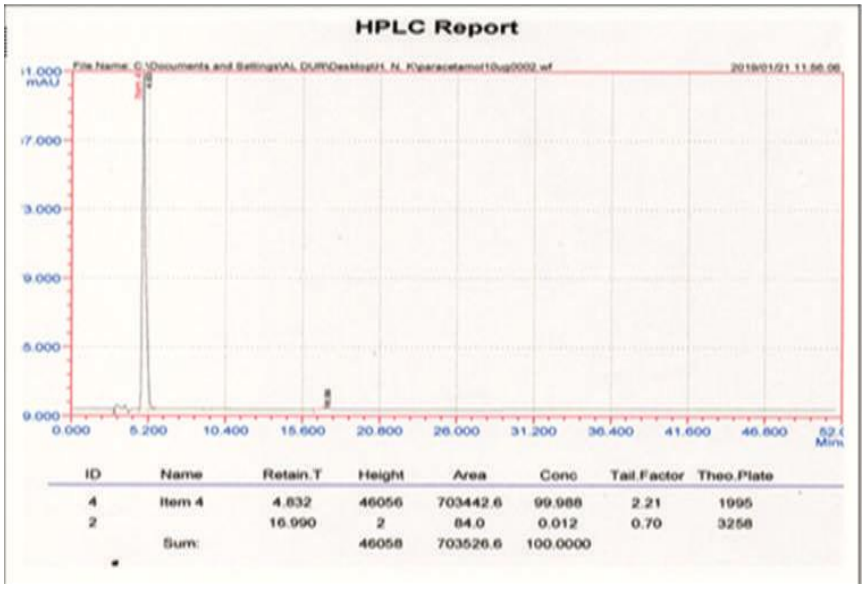
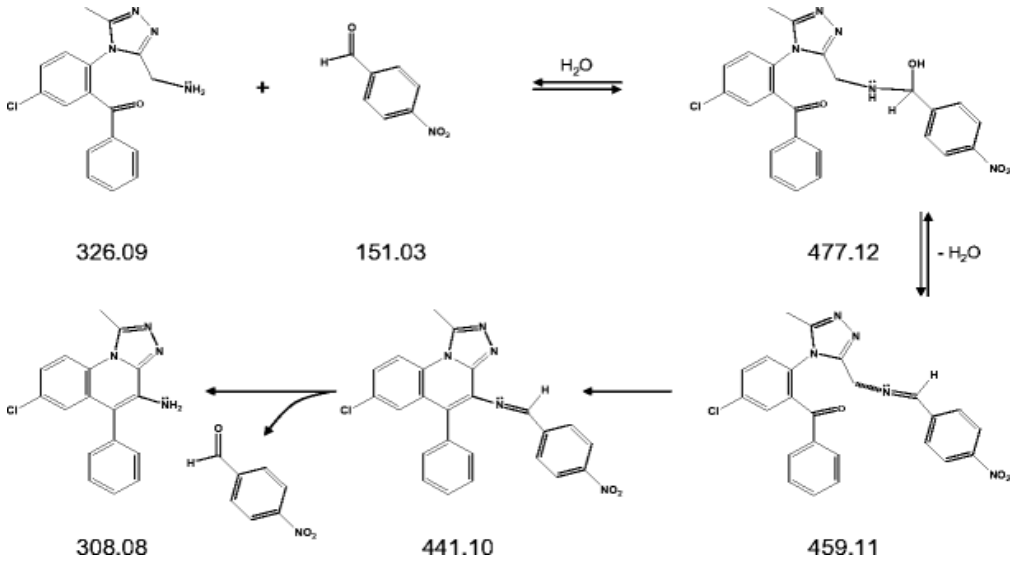


Figure 2: Chromatogram of acid degradation

The base degradation

When using base such as NaOH or potassium hydroxide to suppress ester, products of carboxylic salt, and alcohol. 60 mg from tablet powder of APB was taken in 100 ml volumetric flask. 5 ml of 0.1 N NaOH was added in the flask and kept at 70–80°C reflux condition for 2–3 h. After completion of the stress, the solution was neutralized using 0.1 N HCl and completed up to the mark with mobile phase [Schame 3 and Figure 2].



Schame 3: mechanism of base degradation

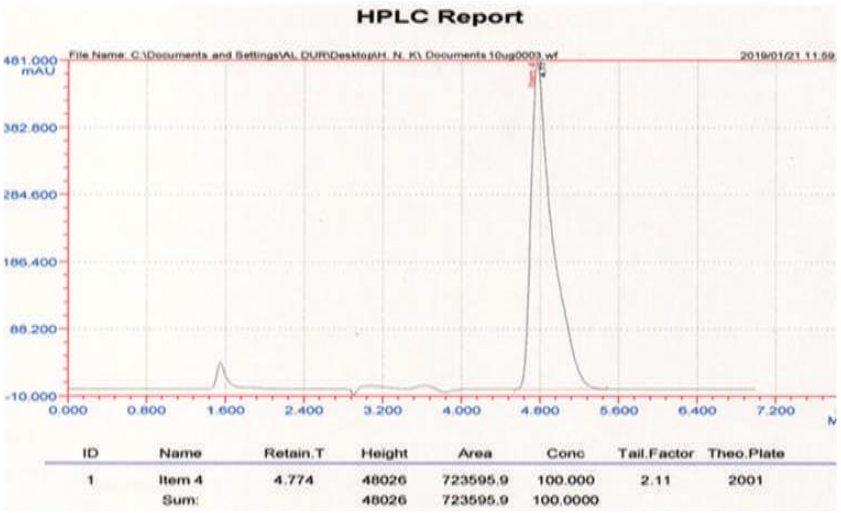


Figure 3: Chromatogram of base degradation

Oxidative degradation

About 60 mg from tablet powder of APB and 5 ml of 20% H_2O_2 were added in 100 ml volumetric flask. The flask was kept at 70–80°C reflux condition for 2–3 h. After

completion of the stress, the flask was completed up to the mark with mobile phase (Figure 4).

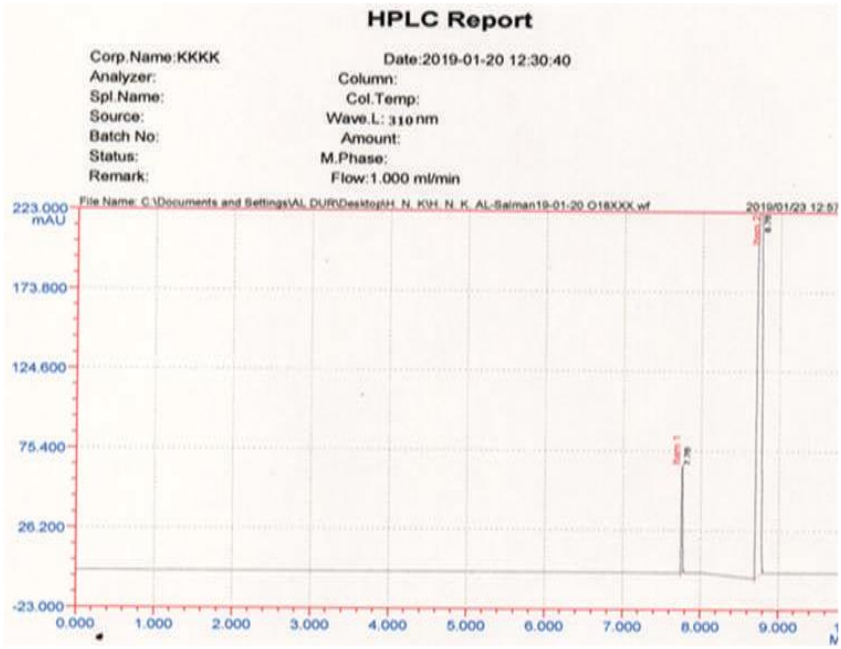


Figure 4: Chromatogram of oxidative degradation

Photolytic degradation

For photolytic degradation study, 60 mg from tablet powder of APB benzoate was transferred into a glass Petri dish and placed in the direct sunlight for 2–3 h. After completion of the stress, the tablet powder was transferred to a 100 ml volumetric flask and made up to the mark with mobile phase. The infrared spectrum of the solution is then analyzed. The process of decomposition in this way leads to the partial disintegration of the APB compound and the uncontrolled interference with pharmaceutical additives and this is evident in Figure 5, where the peaks of HPLC-UV appear irregular and sometimes overlapping.

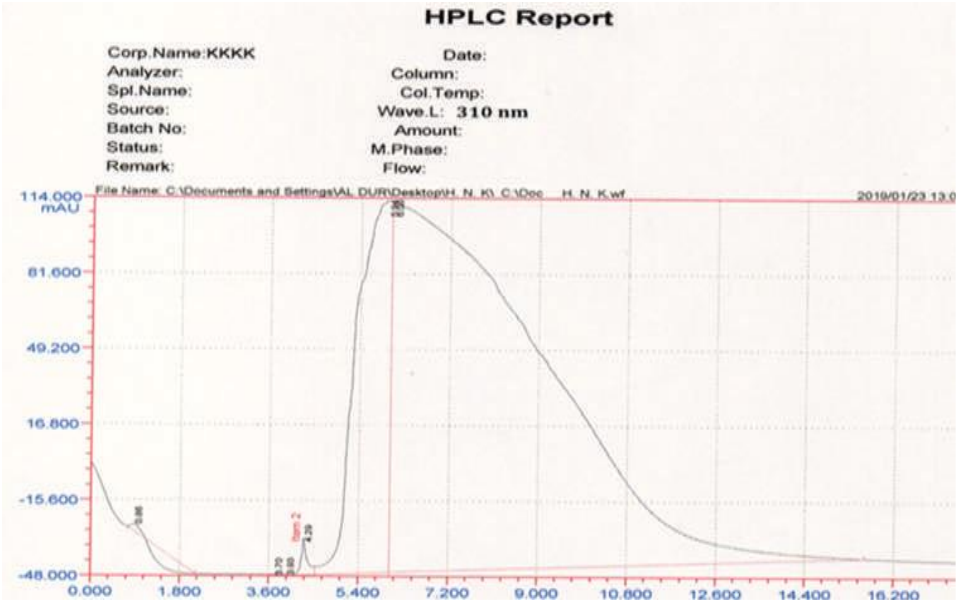


Figure 5: Chromatogram of photolytic degradation

Thermal degradation

For this, 60 mg from tablet powder of APB was taken in glass Petri dish and placed in hot air oven at 105°C for 2–3 h.

After specified time, the tablet powder was transferred to a 100 ml volumetric flask and made up to the mark with mobile phase. Increasing the temperature of the APB solution >100°C indicates that it is difficult to control the synthetic structure of the APB and thus obtain complete thermal dissolution of the compound, this is shown in Figure 6.

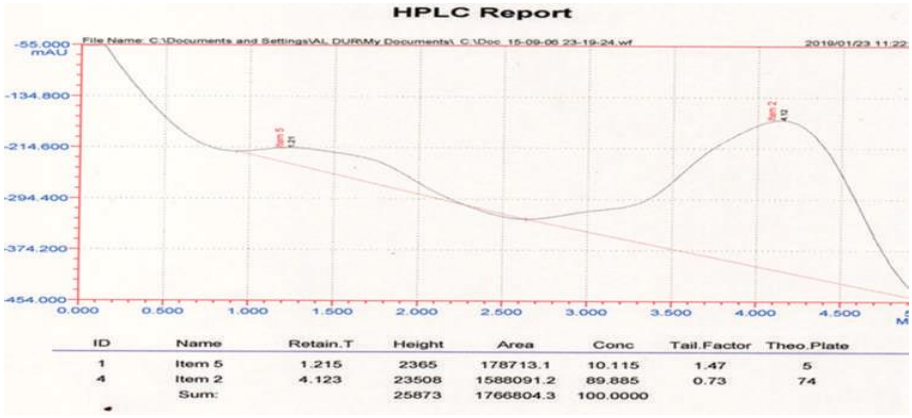


Figure 6: Chromatogram of thermal degradation

INFRARED SPECTRUM OF Alprazolam

In the FT-IR measurements, when comparing the values of the active groups of the standard substance APB with the FT-IR values of the model, we notice a clear convergence between the values, and this indicates the success of the process of isolating the active substance from the drug form by the chromatographic method adopted in this work.

For Pure APB Powder

In the infrared spectrum of standard APB [Figure 7], The characteristic absorption bands at -C-H (Aromatic, Med) 3300 cm^{-1}, -C-H (strach, strong) 2850-3000 cm^{-1}, -C-H (Bending, Varible) 135-1450 cm^{-1}, =C-H (strach, Med) 3010-3100 cm^{-1}, =C-H (Bending, strong) 675-1000 cm^{-1}, -C=C- (strach, Med) 1620-1650 cm^{-1}, -C-Cl (strach, strong) 600 cm^{-1}, -C-N (strach, Med) 1080-1360 cm^{-1}, -C-N (strach, Med) 2210-2260 cm^{-1}-C=N (strach, Med) 1500-1700 cm^{-1}, in the structure of APB, are stored in the test standard APB [24,25].

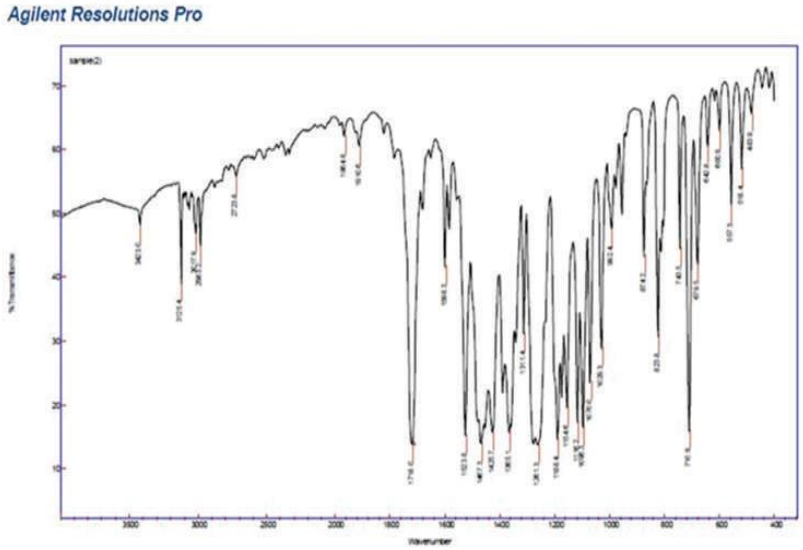


Figure 7: FT-IR spectrum of standard APB

APB and its Crud

The characteristic absorption bands at -C-H (Aromatic, Med) 3150 cm^{-1}, -C-H (strach, strong) 3000 cm^{-1}, -C-H (Bending, Varible) 1404 cm^{-1}, =C-H (strach, Med) 3008 cm^{-1}, =C-H (Bending, strong) 726 cm^{-1}, -C=C- (strach, Med) 169 cm^{-1}, -C-Cl

(strach, strong) 586 cm^{-1}, -C-N (strach, Med) 1026 cm^{-1}, -C-N (strach, Med) 2222 cm^{-1}, -C=N (strach, Med) 1581 cm^{-1}, in the structure of APB, are stored in the test samples (Figure 8).

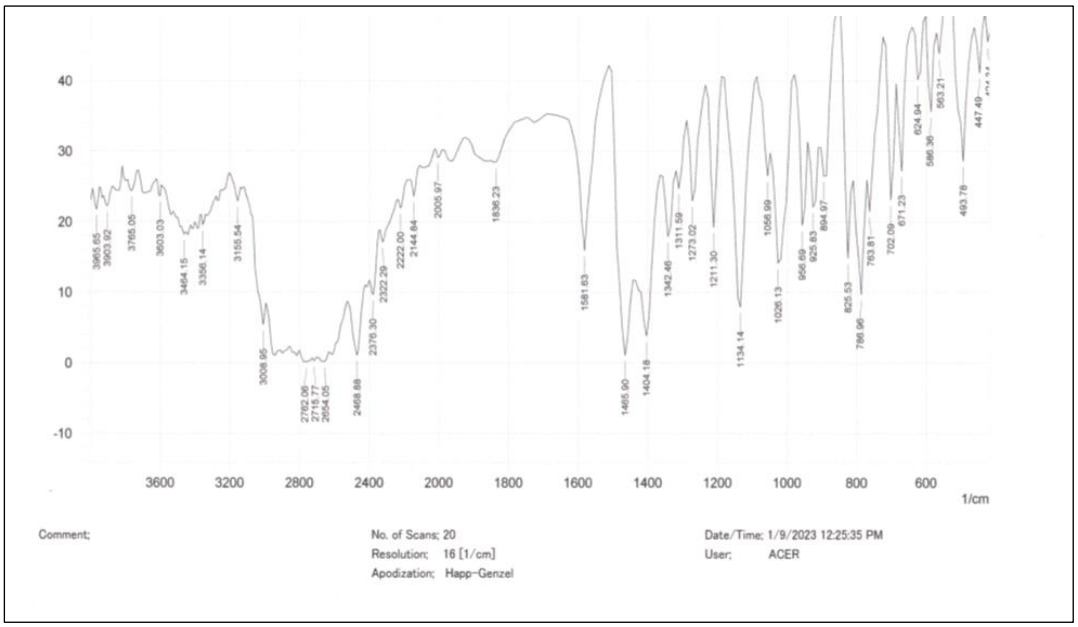


Figure 8: FT-IR spectrum of APB Crud

DISCUSSION OF THE RESULTS

The Optimization of HPLC conditions

The chromatographic conditions were developed to separate all the degradation products from the peaks of APB.

During the process of HPLC method optimization, several trials were taken using Ion Pac Arcus EP-C18; 5 µm, 4.5 mm × 250 mm, with the use of suitable organic phase, acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 and 1 ml/min flow rate. The wavelength was monitored at 310 nm. The retention time for APB was 1.10 min. Good peak shape was observed of the new analytical method (Figure 2).

The System Suitability

Studies were carried out for the purpose of adapting the HPLC-UV system. The standard APB (3 µg/mL) was used through three replicas of the same concentration that was replicated using the optimal method. Table 2 shows the system suitability.

These results meet the requirements of separation method and Alprazolam estimates in various pharmaceuticals.

Table 2: System suitability analysis of APB

Injections	Drug	RT	Area	% area	USP plate count	USP tailing
1	APB	1.10	25000	99.400	3326	1.10
2	APB	1.105	61245	99.540	3543	1.10
3	APB	1.14	11330	99.000	3541	1.10
4	APB	1.14	22375	99.318	3678	1.10
5	APB	1.15	60600	99.425	3125	1.10
MEAN			36110	RT- Retention Time 1.10 ± 0.041 min		
SD			0.38			
% RSD			0.30			

The Validation of Method and Assay

In accordance with ICH guidelines.[26], the new chromatographic method HPLC-UV and parameters such as specificity, linearity range and sensitivity, regression, precision, accuracy, and rigidity were used to validate the method used To assess the method validity, the effect experimental conditions on the peak areas of the analytes were examined. The validity of the method was checked at concentration of 3 µg/mL for APB. Table 3-7 summarized all the results. The results revealed that the peak areas for the drugs were unaffected small changes in flow rate, composition of mobile phase, temperature, and detection wavelength, indicating significant validity of the method [27,28].

The Specificity[29,30]

The specificity of the proposed method was studied using the study of forced degradation. The analysis was performed to ensure that the proposed method was able to separate APB from the potential degradation products generated during the study of forced degradation.

Studies were performed using acid, base, oxidation, photolysis, and heat for the tablet sample at a concentration of 3 µg/ml of APB. Chromatograms shapes are shown in Figures 2-6. The highest percentage of deterioration occurred under the alkaline conditions of the drug. The lowest percentage of the degradation of APB

occurred in the case of thermal and in the case of photosynthesis. One peak degradation was observed in decomposition products. Other degradation products due to stress do not interfere with the detection of APB, so the method can be considered as an indicator of stability.

The Linearity Range and Sensitivity[31,32]

Under the optimum experimental conditions, a linear relationship was established by plotting the peaks areas for drug against the drug concentration (µg/mL). The concentration range was found to be 1 µg/mL to 5 µg/mL for APB. The linear regression analysis of the data gave from the following equations: y = 120130x + 933.5 (R^2 = 0.9998) for APB On the assumption that: y = peak area, x= concentration of the drug (µg/mL), and R^2 = Regression coefficient. The high values of regression coefficients with small intercept indicate the good linearity of the calibration curve that shows in Figure 8 and table 3.

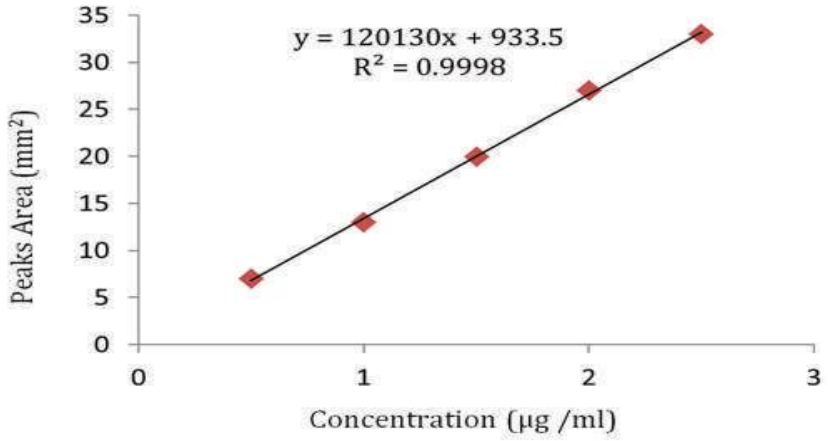


Figure 8: Linearity of the calibration curve

Table: 3 linearity of APB

Sl. No.	Concentration µg/ml	Area
1	1	25000
2	2	61245
3	3	11330
4	4	22375
5	5	60600

The Regression[33,34]

The sensitivity of the proposed method was assessed by calculating limit of quantitation (LOQ) and limit of detection (LOD). The LOD and LOQ were calculated as

follows: LLOQ=10 × SD/S; LLOD= 3.3 × SD/S Where, SD = standard deviation of the drug response and S = Slope of the calibration curve. LLOD values were found to be 0.0143 µg/ml while LLOQ values were found to be 0.0316 µg/ml. These values demonstrate the satisfactory.

sensitivity of the proposed method for the analysis of selected drug. Table 6 shows the results of regression statistics of the proposed method (Table 4).

Table: 4 Regression characteristics of linearity of APB

Parameters	Results
Linearity range (µg/ml)	1- 5
Regression equation (y=mx+b)	y = 120130x + 933.5
Slope (m)	120130
Intercept (b)	933.5
The correlation coefficient (R^2)	0.9998
limit of detection (LOD)	0.0143
limit of quantitation (LOQ)	0.0316

The Accuracy[35,36]

For the pre-analysis tablet sample solutions, a known amount of standard solution was added at three different levels, 10%, 20%, and 30%. The solutions were reanalyzed by the proposed method. The percentage recovery was between 100% and 100% with percentage RSD≤1.0%. The results indicate good accuracy of the method. The selectivity of the method was demonstrated by the non-interference of the excipients with the analysis of the analytes. The results are summarized in Table 5.

Table. 5 Recovery study results of APB

Sl. No.	Accuracy range	Amount of APB added (mg)	Amount recovered (mg)	% Recovery
1.	50 % Accuracy	50	49.5	99.0
2.		50	49.6	99.2
3.		50	49.4	98.8
4.	100% Accuracy	100	100	100
5.		100	100	100
6.		100	100	100
7.	150% Accuracy	150	150	100
8.		150	150	100
9.		150	150	100
Mean				100
SD				0.38
% RSD				0.30

The Precision[37,38]

The precision was established by analyzing APB at a concentration of 3 µg/ml. The system precision was tested by applying the developed method for the determination of APB in the pure standard APB for three successive times (n = 3). The method precision was tested by repeated analysis of APB in tablet sample for three successive times (n = 3). The results are summarized in Table 6 and 7. The percentage RSD values for system precision and method precision were ≤0.01%, indicating that the proposed method has good precision in the analysis of APB.

Table. 6 Method precision

SL. NO.	Sample weight (mg)	Area	Mean Area Counts	% Label Claim
1	100	11330	36110	100
2	100	11330	36110	100
3	100	11330	36110	100
4	100	11330	36110	100
5	100	11330	36110	100
MEAN				100

SD	0.38
% RSD	0.30

Table. 7 Intermediate precision

SL. NO	Sample weight (mg)	Area	Mean	% Label Claim
1	100	114330	36110	100
2	100	114330	36110	100
3	100	114330	36110	100
4	100	114330	36110	100
5	100	114330	36110	100
6	100	114330	114330	100
Mean				100
SD				0.38
% RSD				0.30

THE APPLICATIONS OF METHOD [39-41]

The analytical method of APB was assessed by examining commercially available tablets (Alprazolam comprimate, LPH, 0.25 mg, Labormed Pharmaceutical Industries Limited, syria., that claiming to contain 0.25 mg of APB). The percentage of APB was found where the values were 100 ± 0.300%, while the ratio of APB was found where the values were 100 ± 0.01%. This result indicating the values of percentage recovery and RSD% that the proposed method was accurate and precision in APB analysis in dosages forms. Table 8, summarized the applications results.

Table 8: Assay of APB in tablets

Analyte	Labeled claim (mg)	Found (mg)	Mean (mg)	%Recovery	%RSD
Standard - APB	0.25	0.25	0.25	100	±0.300
APB -0.25	0.25	0.25	0.25	100	±0.302

CONCLUSION

This work described HPLC system (LC100 Angstrom advanced) equipped with a UV detector for APB determination in two commercial pharmaceutical drugs. This developed method considered as simple, inexpensive and needs only a very small volume of the sample as well as used it is an ultraviolet detector makes this system very specific due to one peak in the chromatogram. In this application, there is no need for high sensitivity since the pharmaceutical drugs have a very low concentration. The method was validated as per the HPLC-UV guidelines and the developed method obeys Beer's law over the concentration range of 1.0–5.0 µg/mL for drugs.

Based on the results, this study divulges with important analytical method used to determine the presence of APB in the dosage form. The developed and validated stability-indicating HPLC-UV method for the quantification of APB is simple, accurate, precise, sensitive, specific, rugged, and robust. The proposed method can, thus, be applied for routine analysis of APB in tablet dosage form.

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COMPETING INTERESTS DISCLAIMER

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