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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) METHOD FOR ATENOLOL AND HYDROCHLORTHIAZIDE IN BULK DRUG AND TABLET FORMULATION

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ABSTRACT: A new simple, rapid, precise, accurate, specific and stability indicating assay method was developed for simultaneous estimation of Atenolol (ATN) and Hydrochlorthiazide (HCZ) in pure form and tablet form. The analytes were separated by RP HPLC on a Hypersil-BDS C_{18} column (250X4.6mm i.d., 5 µm).The mobile phase was 25mMphosphate buffer ($_{P}$H 3±0.05): acetonitrile (85:15, v/v) at 0.7 mL/min satisfactorily resolved the binary mixture. The UV detector was operated at 227 nm for the determination of both the drugs. Linearity, accuracy and precision were found to be acceptable over the concentration ranges of 4-48 µg/ml and 1-12 µg/ml for ATN and HCZ, respectively, in binary mixture. The optimized methods proved to be specific, robust and accurate for the quality control of ATN and HCZ in bulk drug and pharmaceutical formulations.

KEY WORDS: Atenolol, Hydrochlorthiazide, Validation, Forced Degradation, Stability Indicating assay

1. INTRODUCTION

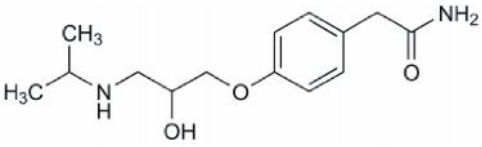
Drugs play a vital role in the progress of human civilization by curing diseases. Today a majority of the drugs used are of synthetic origin. These are produced in bulk and used for their therapeutic effects in pharmaceutical formulations. Pharmaceutical product quality is of vital importance for patient safety. Stability testing of an active substance or finished product provide evidence on how the quality of a drug substance or drug product varies with time influenced by a variety of environmental factors such as pH, temperature, humidity and light. Knowledge from stability studies enables understanding the long-term effects of the environment on the drugs. Stability testing provides information about degradation mechanisms, potential degradation products, possible degradation pathways of drug as well as interaction between the drug and the excipients in drug product [1].

The parent drug stability test guideline Q1A(R2) issued by International Conference on Harmonisation (ICH) requires that stress testing on the drug substance should be carried out to establish its inherent stability characteristics and for supporting the suitability of the proposed analytical procedures [2]. It is suggested that stress testing should include the effect of temperature, humidity, light, oxidizing agents as well as susceptibility across a wide range of pH values.

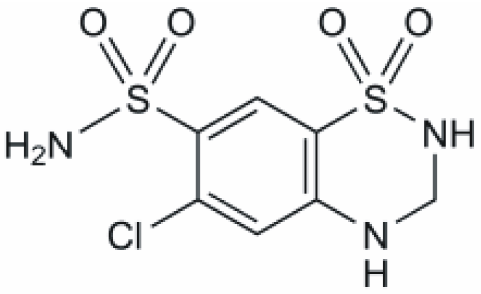
Atenolol is a β_1 receptor specific antagonist, chemically (RS)-4-(2-hydroxy-3-isopropylaminopropoxy) phenylacetamide (Figure- 1) while Hydrochlorthiazide is a diuretic agent, chemically described as a 6-Chloro-3, 4-dihydro-2*H*-1, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide (Figure 1) [3,4]. Atenolol in combination with

hydrochlorthiazide is used in treatment of hypertension. Several methods are available in the literature for the determination of ATN and HCZ. Most of these methods are for the determination of either ATN or HCZ separately, or in combination with other drug. Analytical methods reported for quantitative determination of ATN individually or in combination with other drugs in pharmaceutical formulations or biological fluids are HPLC [5,6,7,8], gas liquid chromatography [9], capillary zone electrophoresis [10], titrimetry [11] and Spectrophotometry [12]. Many analytical methods for HCZ alone or in combination with other drugs including spectroscopic and chromatographic methods are also reported in literature [13-19]. Analytical methods reported for the simultaneous estimation of ATN and HCZ are based on the Spectrophotometry [20,21], LC [22] and chemometrics [23]. But none of these methods are stability indicating. A stability indicating LC-MS [22] method is available in literature. So the aim of the present study was to develop a simple validated stability indicating analytical method according to ICH recommended guidelines [24,25,26].

Figure 1 Chemical structures of Atenolol (ATN) and Hydrochlorthiazide (HCZ)



ATN



HCZ

2. EXPERIMENTAL

2.1 Chemicals and reagents

ATN and HCZ reference standards were kindly gifted by M/s Cadila Pharmaceuticals Ltd, Ahmedabad. Commercial pharmaceutical preparation Atenova-H (M/s Cadila Pharmaceuicals Ltd.) containing 50mg of ATN and 12.5mg of HCZ was collected from local market. Acetonitrile, methanol and water used were of HPLC grade (Qualigens Fine Chemicals, Mumbai, India). Ortho-phosphoric acid was AR grade (Qualigens Fine Chemicals, Mumbai, India). A 0.2 µm nylon filter (Pall life Sciences, Mumbai, India) was used. All other chemicals and reagents used were analytical grade unless otherwise indicated.

2.2 Apparatus

The chromatographic system (Shimadzu corporation, Kyoto, Japan) consisted of LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20 µL fixed loop and a SPD-20A Prominence UV-visible detector. The separation was performed on a Hypersil BDS C_{18} column (Thermoquest, Mumbai, India) at an ambient temperature. Chromatographic data were recorded and processed using Spinchrom Chromatographic Station CFR version 2.4.0.193 (Spinchrom Pvt. Ltd., Chennai, India). An ultrasonicator DTC 503 (Ultrasonics Selec, Vetra, Italy) was used for degassing the mobile phase.

2.3 Chromatographic Conditions

Chromatographic separations of active (ATN and HCZ) and related substances (Degradation products) were obtained by using Hypersil-BDS C18 column (250X4.6mm i.d., 5 µm particle size) from Thermo-quest. Mobile phase 25mM Phosphate buffer pH 3.0±0.05: Acetonitrile (85:15, v/v) (PH 3 was adjusted with O-Phosphoric acid) was prepared, filtered through a 0.2 µm nylon filter and degassed for 5 min in an ultrasonicator. The mobile phase was pumped through the column at flow rate of 0.7 mL/min. Analyses were carried out at ambient temperature with detection at 227 nm. The injection volume was 20 µL and each analysis required 12 min.

2.4 Standard Solutions

Stock standard solutions of ATN 1 mg/mL and HCZ 1 mg/mL were prepared by dissolving 50 mg ATN standard and 50 mg HCZ standard in 50 mL methanol. Working standard solutions of ATN 0.1 mg/mL and HCZ 0.1 mg/mL were prepared by diluting suitable aliquots of corresponding stock solutions with mobile phase.

2.5 Sample Solution

Twenty Atenova-H tablets were accurately weighed and finely powdered. An accurately weighed amount equivalent to 12.5 mg of HCZ and 50 mg ATN was transferred into 50 mL volumetric flask, dissolved in 20 ml of methanol and volume was made up to the mark with the same solvent. The solution was stirred for 10 min using a magnetic stirrer and filtered into a 50 mL volumetric flask through 0.45µm nylon membrane filter. The residues were washed 3 times with 5 mL of methanol, and then the volume was made up to 50 mL with the same solvent. This solution was diluted to 1:10 with mobile phase and final dilution was done in mobile phase to give final concentration of 6 µg/mL and 24 µg/mL of HCZ and ATN, respectively.

2.6 Forced Degradation of ATN & HCZ

2.6.1 Stock Solutions- Stock solutions of binary mixture of ATN and HCZ was prepared in according to the ratio in the formulation (1:4 for HCZ and ATN, respectively). Stock solution of formulation Atenova-H was also prepared in methanol

2.6.2 Acidic Hydrolysis- To 10 ml of above methanolic stock solutions of binary mixture and pharmaceutical formulation, 10 ml of 1 M HCl, was added separately. These mixtures were refluxed separately for 1 hour at

80ºC on oil bath. The forced degradation in acid media was performed in the dark in order to exclude possible photo-degradation. The degradation samples were then cooled to room temperature. Suitable aliquots of resultant degradation samples were taken and neutralized for assay after suitable dilutions with mobile phase.

2.6.3 Alkaline Hydrolysis- To 10 ml of above methanolic stock solutions of binary mixture and pharmaceutical formulation, 10 ml of 0.5 M NaOH, was added separately. These mixtures were refluxed separately for 1 hour at 80ºC on oil bath. The forced degradation in alkaline media was performed in the dark in order to exclude possible photo-degradation. The degradation samples were then cooled to room temperature. Suitable aliquots of resultant degradation samples were taken, neutralized and subjected to analysis after suitable dilutions with mobile phase.

2.6.4 Oxidative Hydrolysis- To 10 ml of above methanolic stock solutions binary mixture and pharmaceutical formulation, 10 ml of 3 %v/v H_2O_2 was added separately. These mixtures were refluxed separately for 1 hour at 80ºC on oil bath. The forced degradation in oxidative media was performed in the dark in order to exclude possible photo-degradation. The degradation samples were then cooled to room temperature. Suitable aliquots of resultant degradation samples were taken and subjected to analysis after suitable dilutions with mobile phase.

2.6.5 Neutral Hydrolysis- To 10 ml of above methanolic stock solutions of binary mixture and pharmaceutical formulation, 10 ml of water was added separately. These mixtures were refluxed separately for 6 hours at 80ºC on oil bath. The forced degradation in neutral media was performed in the dark in order to exclude possible degradation effect of light. The degradation samples were then cooled to room temperature. Suitable aliquots of resultant degradation samples were taken and subjected to analysis after suitable dilutions with mobile phase.

2.6.6 Dry Heat Degradation- For dry heat degradation, binary mixture and pharmaceutical formulation were placed in oven at 80ºC for 24 hours under dry heat condition in the dark and then cooled to room temperature. Degradation samples were subjected to analysis after suitable dilutions with mobile phase.

2.6.7 Wet Heat Degradation- The above stock solutions of binary mixture and pharmaceutical formulation were refluxed separately for 4 hours at 80ºC on oil bath for wet heat degradation. The degradation samples were then cooled to room temperature. Suitable aliquot of resultant degradation samples were taken and subjected to analysis after suitable dilutions with mobile phase.

2.6.8 Photochemical Degradation- The photochemical stability of drug was studied by exposing stock solutions of binary mixture and formulation in direct sunlight for 3 days (from 10-00 am to 06-00 pm) on wooden plank and kept on terrace. Degradation samples were subjected to analysis after suitable dilutions with mobile phase.

2.7 Validation of Proposed Method

2.7.1 Calibration curve (linearity)- Accurately measured aliquots of working standard solutions equivalent to 4-48 µg ATN and 1-12 µg HCZ were transferred to 2 series of 10 mL volumetric flasks and the contents of the flasks were diluted to volume with mobile phase. A 20 µL aliquot of each solution was injected in triplicate into the liquid chromatograph. The conditions including the flow rate of mobile phase at 0.7 mL/min, detection at 227 nm and run time program for 12 min, were adjusted. A calibration curve for each drug was obtained by plotting area under the peak (AUP) versus concentration (C).

2.7.2 Accuracy (% recovery)- The accuracy of the methods was determined by calculating recoveries of ATN and HCZ by the standard addition method. Known amounts of standard solutions of ATN (6.4, 8.0, and 9.6 µg/mL) and HCZ (1.6, 2.0, and 2.4 µg/mL) were added to prequantified sample solutions of tablet dosage forms. The amounts of ATN and HCZ were estimated by applying these values to the regression equations of the calibration curves.

2.7.3 Method precision (repeatability)- The instrumental precision was checked by repeatedly injecting (*n=5*) solution of binary mixture containing ATN (24 µg/mL) and HCZ (6 µg/mL).

2.7.4 Intermediate precision (reproducibility)- The intraday and interday precisions of the proposed method were determined by estimating the corresponding responses 5 times on the same day and on 5 different days for 3 different concentrations of ATN (8, 24, and 40 µg/mL) and HCZ (2, 6, and 10 µg/mL). The results are reported in terms of relative standard deviation (RSD).

2.7.5 Limit of detection (LOD) and limit of quantitation (LOQ)- LOD and LOQ of the drug were calculated using the following equations according to International Conference on Harmonization (ICH) guidelines

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

where σ = the standard deviation of the response and S = the slope of the regression equation.

2.7.6 Robustness- The robustness of the method is its ability to remain unaffected by small changes in parameters. Effect of two different brands of acetonitrile on the retention time and slight change in flow rate were applied as variable parameters. Flow rate varied at three levels (-1, 0, 1). One factor at a time was changed to estimate the effect. Thus replicate injections (n=3) of standard solution at three concentration levels were performed under small changes of two chromatographic parameters (factors).

2.7.7 Specificity- Specificity is the ability of the analytical method to measure analyte response in presence of interferences including degradation products and related substances. Specificity was checked by determining ATN and HCZ in laboratory prepared binary mixture and in binary mixture containing different degradation products.

2.7.8 System suitability Test (SST)- In the system suitability test binary solution of 40 µg/ml of ATN and 10 µg/ml of HCZ (n=5) was prepared and injected. Then the system suitability parameters like retention time, theoretical plates, tailing factor and resolution were calculated from the chromatogram.

2.7.9 Analysis of ATN and HCZ in Combined Tablet Dosage Form- Tablets containing ATN (50 mg) and HCZ (12.5 mg) of the brand Atenova-H, from M/S Cadila Pharmaceuticals Ltd, Ahmedabad, India, were purchased from the local market. The responses of the tablet dosage form were measured at 227 nm for quantification of ATN and HCZ by using LC method above. The amounts of ATN and HCZ present in sample solutions were determined by adjusting the responses into the regression equations for ATN and HCZ.

3. RESULTS AND DISCUSSION

The absorption spectra of ATN and HCZ greatly overlap; so conventional determination of these compounds in mixture is not possible. To optimize the LC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for ATN and HCZ were obtained with a mobile phase consisting of 25mM Phosphate buffer PH 3.0±0.05: Acetonitrile (85:15, v/v). Quantification of the drugs was performed at 227 nm. Resolution of the components with clear baseline separation was obtained (Figure 2).

3.1 Validation of the Proposed Method

3.1.1 Linearity- Linear correlation was obtained between peak areas and concentrations of ATN and HCZ in range of 4–48 and 1–12 µg/mL, respectively. The linearity of calibration curves was validated, and correlation coefficients of regression were found near to 1 (Table- 1). The results show that good correlation existed between the peak area and concentration of the analytes.

3.1.2 Accuracy- The recovery experiments were performed by the standard addition method. The recoveries obtained were 100.30 ± 0.85 and 100.14± 0.89% for ATN and HCZ, respectively (Table 2). The high values indicate that the method was accurate.

3.1.3 Method precision- The RSD values for ATN and HCZ in combined formulations were found to be 0.66 and 0.72%, respectively (Table 2). The low RSD values indicate that the proposed method is repeatable.

3.1.4 Intermediate precision- The intraday RSD values for ATN and HCZ were 0.413–0.812 and 0.473–0.799%, respectively. The interday RSD values for ATN and HCZ were 0.691–0.762 and 0.239–0.938%, respectively. The % RSD (< 2%) values indicate that the method was sufficiently precise (Table 2).

3.1.5 LOD and LOQ- *LOD* values for ATN and HCZ were found to be 26.61 ng/mL and 7.64 ng/mL, respectively. LOQ values for ATN and HCZ were found to be 79.86 ng/mL and 22.89 ng/mL, respectively (Table 2). These data showed that the method was sensitive enough for the determination of ATN and HCZ.

3.1.6 Specificity- Specificity was determined on laboratory prepared binary mixture and in binary mixture containing different types of degradation products (Stability Indicating Assay Method). Good resolution and absence of interference between drugs determined and degradation products is shown in Figures 3,4,5,6,7 and 8. Moreover the proposed method was applied to a pharmaceutical formulation containing two drugs under study (Figure 9). The standard addition technique was used to assess the accuracy of proposed method. Satisfactory results were obtained indicating the high specificity of proposed method for determination of ATN and HCZ in binary mixtures or in Binary mixture containing different types of degradation products like acidic hydrolysis, alkaline hydrolysis, oxidative hydrolysis, photo degradation etc. and also in pharmaceutical formulation (Table 2 and 3).

3.1.7 Robustness- Robustness of the method was determined by making slight changes in chromatographic conditions. Effect of two different brands of acetonitrile on the retention time and slight change in flow rate were applied as variable parameters. Flow rate varied at three levels (-1, 0, 1). One factor at the time was changed to estimate the effect. Thus replicate injections (n=3) of standard solution at three concentration levels were performed under small changes of two chromatographic parameters (factors). Results presented in Table 4 indicate that the selected factors remained unaffected by small variation of these parameters. It was also found to that acetonitril of different lots from the same manufacture has no significant influence on the determination of retention time. Insignificant variability in retention time was observed (Table 4) indicating the robustness of the method.

3.1.8 System Suitability Test- A binary solution of 40 µg/ml of ATN and 10 µg/ml of HCZ (n=5) was prepared and same was injected, then the system suitability parameters were calculated from the chromatogram. The parameters, retention times, resolution factor, tailing factor and theoretical plates were evaluated. The results (Table 5) obtained from system suitability tests are in agreement with the USP requirements.

CONCLUSIONS

The proposed LC method presented in this paper has advantages of simplicity, accuracy, precision and convenience for separation and quantitation of ATN and HCZ in combination and can be used for the assay of their respective dosage form. Moreover, the proposed LC method is a stability indicating assay method that can determine ATN and HCZ in presence of their degradation products. Thus, the proposed LC method can be used for the quality control of ATN and HCZ in typical laboratories.

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The authors are thankful to M/s Cadila Pharmaceuticals Ltd (Ahmedabad, Gujrat, India) for supplying gift samples of Atenolol and hydrochlorthiazide.

Table 1. Regression analysis of the calibration curves for ATN and HCZ in the proposed HPLCMethod

Parameter	*Atenolol*	Hydrochlorthiazide
Linearity Range (µg/mL)	4-48	1-12
Detection Wavelength (nm)	227	227
Slope ± SD	23.8123 ± 0.167	100.6066 ± 0.112
Intercept ± SD	1.1193 ± 0.734	- 0.1606 ± 0.643
Correlation coefficient	0.9992	0.9997

SD- Standard deviation, 3 determinations

Table 2. Summary of the validation parameters for the proposed HPLC method

Parameter	*ATN*	HCZ
LOD[a]	26.61 ng/mL	7.64 ng/mL
LOQ[b]	79.86 ng/mL	22.89 ng/mL
Accuracy,%	99.9-100.67	99.96-100.30
Repeatability (RSD[c], %; *n* = 5)	0.66	0.72
Precision (RSD, %)		
Interday, *n* = 3	0.691–0.762	0.239–0.938
Intraday, *n* = 3	0.413–0.812	0.473–0.799

a LOD = Limit of detection.
b LOQ = Limit of quantification.
c RSD = Relative standard deviation.

Table 3. Assay results for the combined dosage form using the proposed HPLC method

Formulation	*Atenolol ± SD*[a]	Hydrochlorthiazide ± SD[a]
Atenova-H	100.73%±0.68	99.68%±0.51

a SD = Standard deviation, 5 determinations.

Table 4. Robustness evaluation of proposed HPLC method

Chromatographic changes Factor	Level	Retention time (min)	Asymmetric factor
A: flow rate (mL/ min)			
0.6	-1	5.402	10.101
0.7	0	5.455	10.225
0.8	1	5.503	10.290
Mean ± SD (n = 3)		5.45±0.053	10.205±0.096
B: solvents of different brands			
First	-1	5.342	10.203
second	0	5.489	10.150
Mean ± SD (n = 2)		5.41±0.105	10.176±0.037

SD =Standard Deviation

Table 5. System suitability test parameters for ATN and HCTZ for the proposed HPLC method

Parameters	*Atenolol ± % RSD*[a]	Hydrochlorthiazide ± % RSD[a]
Retention Time, min	5.438±0.066	10.193±0.072
Theoretical plates	10056±0.08	10356±0.09
Tailing factor	1.454± 0.02	1.563± 0.03
Resolution	10.342	

[a] RSD = Relative standard deviation.

Figure 2 Typical liquid chromatogram obtained for a 20 µL injection of a synthetic binary mixture of ATN and HCZ

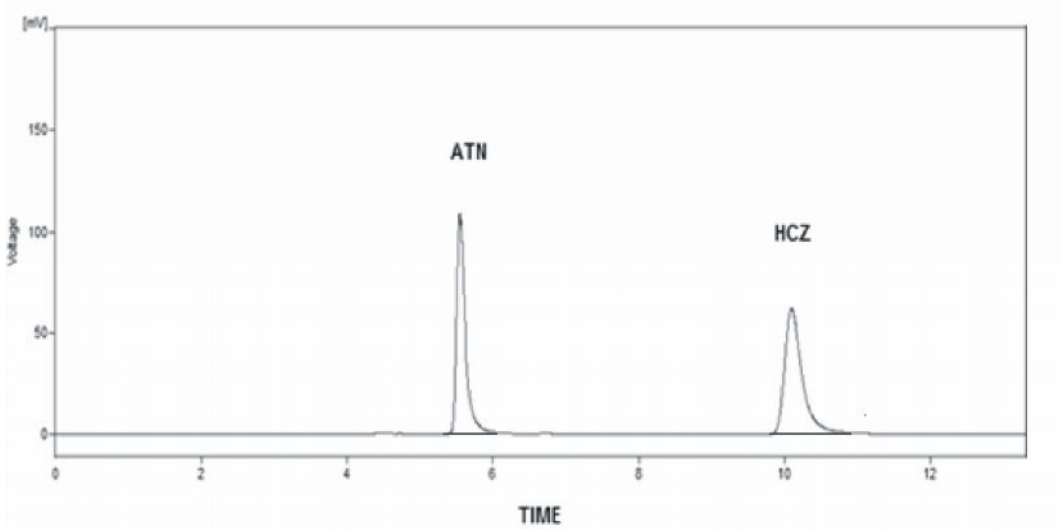


Figure 3 Chromatographic separations of ATN and HCZ from their acidic degradation products

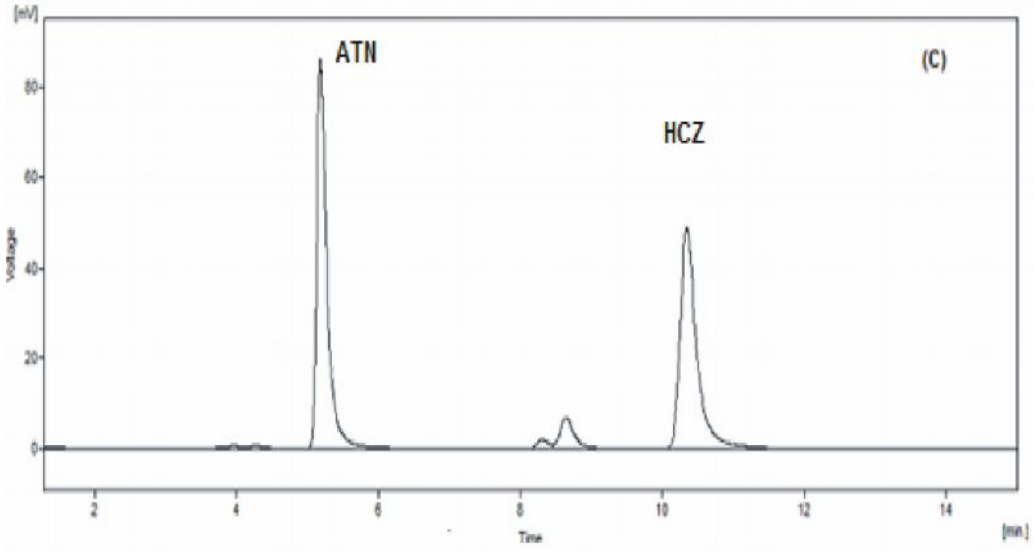


Figure 4 Chromatographic separations of ATN and HCZ from their alkaline degradation products

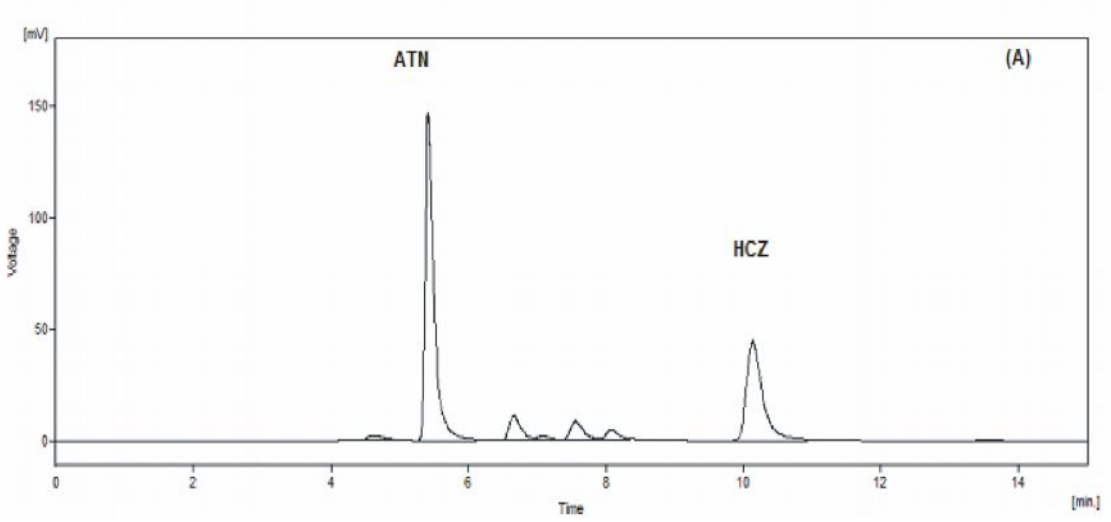


Figure 5 Chromatographic separations of ATN and HCZ from their oxidative degradation products

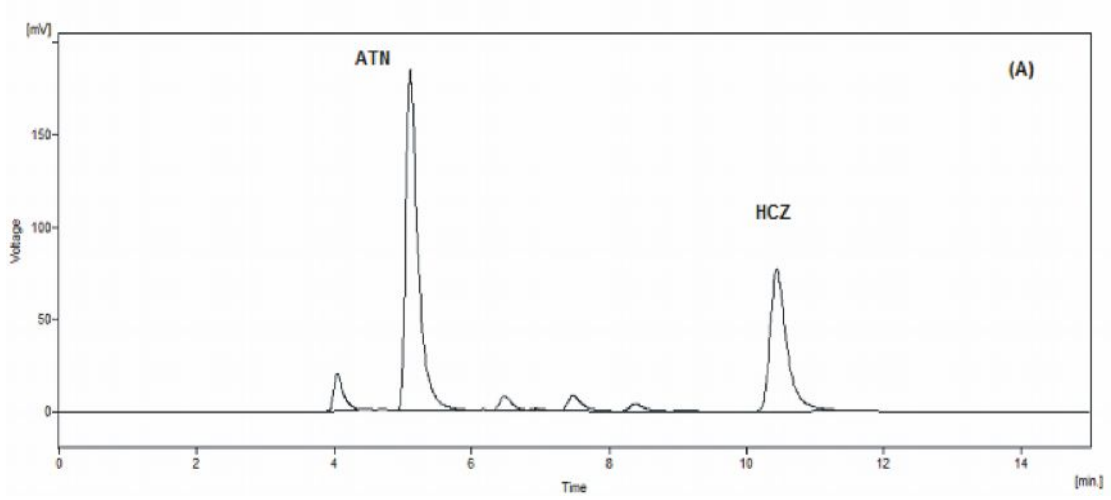


Figure 6 Chromatographic separations of ATN and HCZ from their neutral degradation products

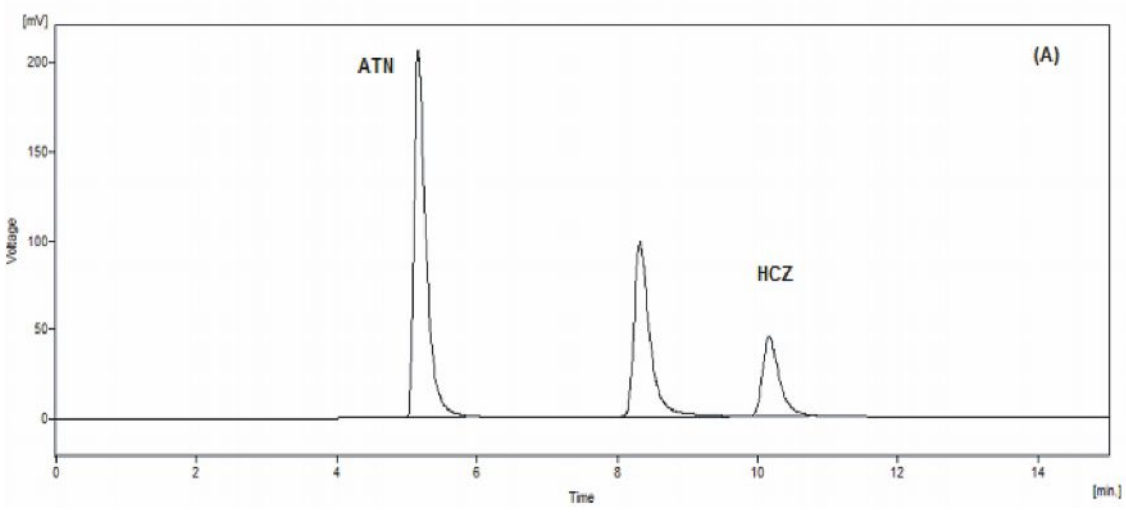


Figure 7 Chromatographic separations of ATN and HCZ from their dry heat degradation products

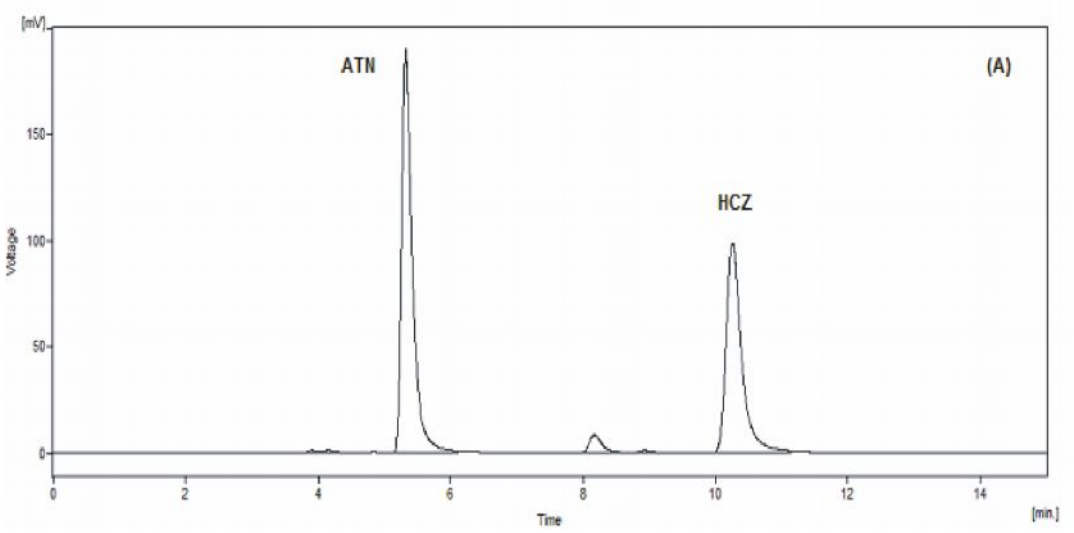


Figure 8 Chromatographic separations of ATN and HCZ from their wet heat degradation products

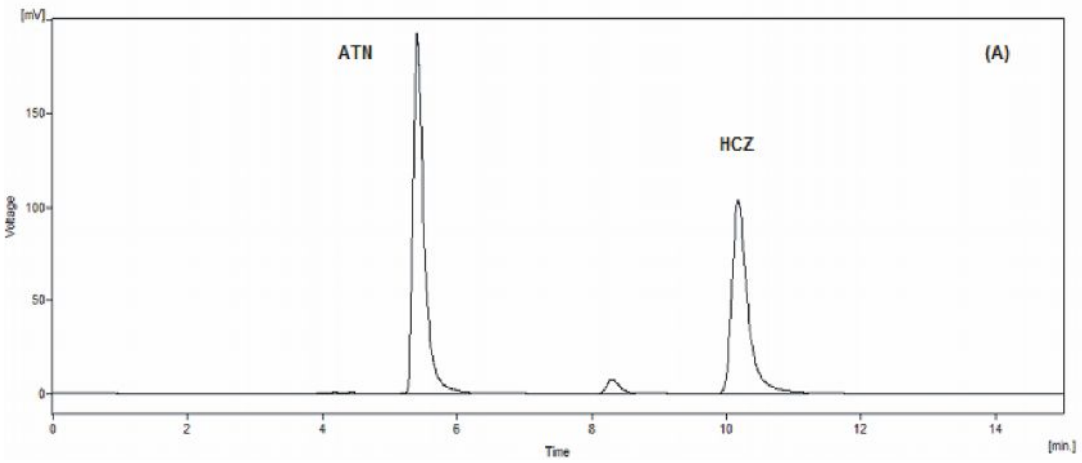
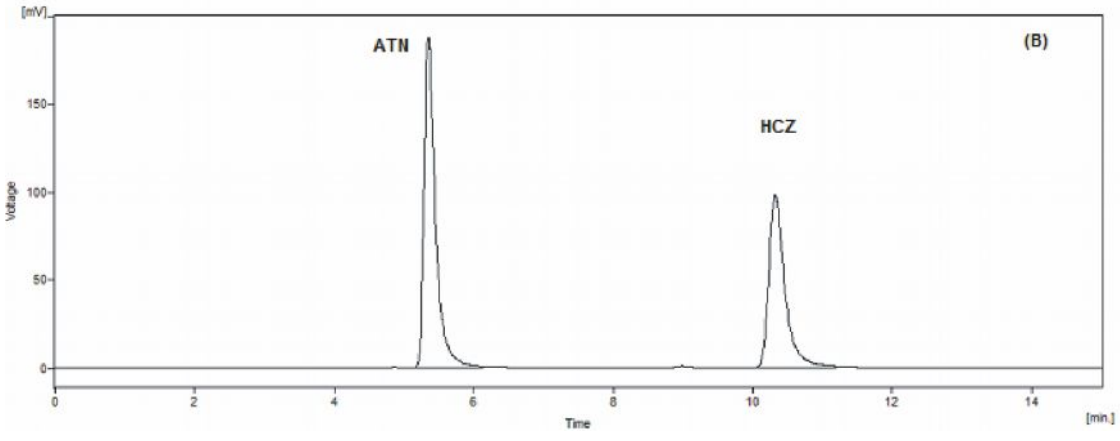


Figure 9 Chromatographic separations of ATN and HCZ from their formulation



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