

**Research Article**

## Development of validated RP-HPLC method for the simultaneous estimation of atenolol and chlorthalidone in combine tablet dosage form

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ATN,  
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Assay method,  
Method Validation

### Abstract

A RP-HPLC method for the estimation of ATN (Atenolol) and CTN (chlorthalidone) in combined dosage form was developed using Comosil RP-C18 (4.6 x 250mm, 5 $\mu$ m) in an gradient mode with mobile phase comprising of Methanol: Water (pH 3 using OPA) The flow rate was 1 mL/ min and effluent was monitored at 226.0 nm. The retention times were found to be 2.2 min for ATN and 3.36 min for CTN. The assay exhibited a linear dynamic range of 40- 200  $\mu$ g/mL for ATN and 10- 50  $\mu$ g/mL for CTN. The calibration curves were linear ( $r^2 = 0.999$  for ATN and  $r^2 = 0.999$  for CTN) over the entire linear range. Mean % recovery was found to be 99.78 % for ATN and 99.30 % for CTN with % RSD was NMT 2 for both estimations which fully agrees with system suitability which is in good agreement with labeled amount of formulation. The % RSD for Intra- Day & Inter-Day Precision was NMT than 2 for both the drugs. The developed method was validated as per ICH guidelines

### 1. Introduction

The technique High Performance Liquid Chromatography (HPLC) is so called because of its improved performance over the classical column chromatography. The technique basically involves the use of porous material as a stationary phase and the liquid mobile phase is pumped into the column under high pressure. The development of this technique is attributed to the small particle size of stationary phase. As the particle size is small the resistance to the flow of mobile phase is very high that is the reason why the high pressure is recommended.<sup>1, 18</sup> Analytical method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. This technical brief will focus on development and validation activities as applied to drug products. Method validation is the process of proving that an analytical method is acceptable for its intended purpose. The parameters for method validation as defined by ICH (International Conference on Harmonization) guidelines are Accuracy, Precision, Specificity, Limit of Detection, Limit of Quantitation, Linearity, Range, Robustness and Ruggedness<sup>2</sup>. From the literature review<sup>7-16</sup> it has been found that only three analytical methods for the above combination have been reported. Therefore the attempt is made to develop simple, accurate, precise rapid and economical RP-HPLC method for determination of Atenolol (ATN) and Chlorthalidone (CTN) in combine dosage form. Atenolol [Figure 1] Chemically is (RS)-2-{4-[2-Hydroxy-3-(propan-2-ylamino)propoxy]phenyl}acetamide. It is white to almost white powder used as anti- hypertensive having solubility in methanol and water, sparingly soluble in ethanol. While chlorthalidone [Figure 2] chemically is (RS)-2-chloro-5-(3-Hydroxy-1-oxoisindolin-3-yl) benzenesulphonamide.<sup>5, 6, 19, 20</sup> It is white to yellowish white crystalline and practically odorless. Used as anti-hypertensive having solubility in methanol and insoluble in water, slightly soluble in ethanol.

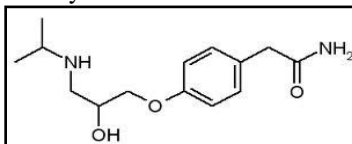


Figure 1 Chemical Structure of Atenolol

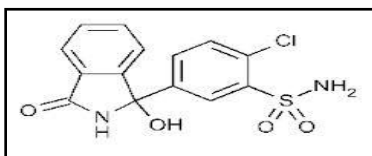


Figure 2 Chemical Structure of Chlorthalidone

## 2. Experimentals

### 2.1 Reagents & Chemicals

Standard samples of ATN & CTN were received as gift samples from the leben laboratories akola (Maharashtra) and IPCA Laboratories mumbai (Maharashtra). The marketed formulation Tenoric (IPCA Laboratories) was purchased from the local market containing ATN 50 mg and CTN 12.5 mg and all the chemicals used were are of analytical grade.

### 2.2 Instruments

HPLC System of Younglin Quaternary pump with UV- VIS detector (190-990 nm) Software – Autochro. Analytical balance of citizen model CY 104 (microanalytical balance) was used for weighing purpose also the ultrasonicator servewell instruments model RC-SYSTEM MU-1700 used for sonication purpose.

### 2.3 Preparation of Standard Solutions

**Standard Stock Solution (A)** Accurately weighed quantity of ATN (40.0 mg) was transferred to 10.0 mL volumetric flask and dissolved in methanol. The volume was made up to mark with methanol to get final concentration of 4000 µg/mL. The resultant solution was then sonicated for 10.0 min in ultrasonicator.

**Standard Stock Solution (B)** Accurately weighed quantity of CTN (10.0 mg) was transferred to 10.0 mL volumetric flask and dissolved in methanol. The volume was made up to mark with methanol to get final concentration of 1000 µg/mL. The resultant solution was then sonicated for 10.0 min in ultrasonicator.

**Working Standard Solution (C)** 0.1 mL of solution (A) and 0.1 mL of solution (B) was transferred to 10.0 mL volumetric flask and then the volume was made up to the mark with mobile phase to get final concentration of (40.0 µg/mL of ATN & 10.0 µg/mL of CTN) respectively. The resultant solution was then sonicated for 10.0 min in ultrasonicator.

### 2.4 Optimization of Mobile Phase and Chromatographic Conditions

**Procedure:** The chromatographic conditions were set as per the optimized parameters. The mobile phase was allowed to equilibrate with stationary phase as was indicated by a steady baseline. Solution (C) was injected in the Rheodyne injector (20.0 µl) and the respective chromatograms were recorded. Various mobile phases were tried by permutations and combinations and also by varying column, flow rate, column temperature and type of buffers with varying pH and solvents. The various mobile phases tried are as follows.

- **Trial 1** Methanol: Water (80: 20) pH 7
- **Trial 2** Methanol: Water (60: 40) pH 7
- **Trial 3** Methanol: Water (50:50) pH 7
- **Trial 4** Methanol: Water (35: 65) pH 7
- **Trial 5** Acetonitrile: Methanol: Water (15: 30: 55) pH 7
- **Trial 6** Methanol: Water (60: 40) pH 3

Above mentioned various mobile phases were tried. The mobile phase containing Methanol: water (60: 40) at pH 3, injection volume-20.0 µL flow rate of 1mL/min was selected, due to its high resolving power, sensitivity and suitability, for the determination of ATN and CTN. The chromatogram is shown in **Figure 1**. Hence the following optimized chromatographic parameters were selected to carry out further experimentation.

- **Column** : Comosil RP-C18 (4.6 x 250mm, 5µm)
- **Flow Rate** : 1 mL/min
- **Wavelength** : 226.0 nm
- **Injection Volume** : 20.0 µL
- **Column Temperature** : Ambient
- **Run Time** : 20.0 min
- **Mobile Phase** : Methanol: Water (60:40)
- **pH** : 3 (Using OPA)

### 2.5 System Suitability Studies

System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be carried out. It is performed to ensure that the system is operating properly and read to deliver results with acceptable accuracy and precision. The tests were performed by collecting data from five replicate injections of standard solutions.

**Procedure:** The chromatographic conditions were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady baseline. Five replicate injections of mixed working standard solution (C) were injected in to the system, the chromatograms were recorded for both the drugs and the results are shown in **Table 1 & 2**.

## 2.6 Analysis of Standard Laboratory Mixtures

### Preparation of Standard Laboratory Mixtures (Standard)

**ATN Standard Stock Solution (A)** Accurately weighed quantity of ATN (40.0 mg) was transferred to 10.0 mL volumetric flask and dissolved in methanol. The volume was made up to mark with methanol to get final concentration of (4000 µg/mL of ATN). The resultant solution was then sonicated for 10.0 min in ultrasonicator.

**CTN Standard Stock Solution (B)** Accurately weighed quantity of CTN (10.0 mg) was transferred to 10.0 mL volumetric flask and dissolved in methanol. The volume was made up to mark with methanol to get final concentration of (1000 µg/mL of CTN). The resultant solution was then sonicated for 10.0 min in ultrasonicator.

**Mixed Standard Solutions** 0.1 mL of solution (A) and 0.1 mL of solution (B) was then transferred to 10.0 mL volumetric flask and volume was made up to the mark with mobile phase to get final concentration of (40.0 µg/mL of ATN & 10.0 µg/mL of CTN) respectively. Similarly 0.2 mL of solution (A) and 0.2 mL of solution (B) was then transferred to 10.0 mL volumetric flask and volume was made up to the mark with mobile phase to get final concentration of (80.0 µg/mL of ATN & 20.0 µg/mL of CTN) respectively. The resultant solutions were then sonicated for 10.0 min in ultrasonicator.

### Preparation of Standard Laboratory Mixtures (Sample)

Accurately weighed 50.0 mg of ATN and 12.5 mg of CTN (as per labeled requirement of marketed formulation) was transferred to 50.0 mL volumetric flask and dissolved in sufficient quantity of methanol. Then the volume was made up to the mark with methanol. The resultant solution was then sonicated in ultrasonicator for 10.0 min. then aliquot portions of 0.4 mL and 0.8 mL was then transferred to two separate 10.0 mL volumetric flask and then volume was made up to the mark with mobile phase to get final concentrations of (40.0 µg/mL & 80.0 µg/mL of ATN and 10.0 µg/mL & 20.0 µg/mL of CTN) respectively. The peak area of standard laboratory mixture and sample laboratory mixture was compared to obtain the concentration. The amount of each drug estimated in laboratory mixture was calculated using following formula –

$$\% \text{ Estimation} = \frac{A_t}{A_s} \times \frac{D_s}{D_t} \times \frac{W_s}{W_t} \times 100$$

Where,

- A<sub>t</sub> = Area count for sample solution
- A<sub>s</sub> = Area count for standard solution
- D<sub>s</sub> = Dilution factor for standard
- D<sub>t</sub> = Dilution factor for sample
- W<sub>s</sub> = Weight of standard (mg)
- W<sub>t</sub> = Weight of sample (mg)

The results are shown in **Table 3**.

## 2.7 Analysis of Marketed Formulation

### Preparation of Standard Solutions

Prepared as per the methodology adopted for laboratory mixtures

### Preparation of Sample Solutions

Ten Tablets were weighed accurately and ground to fine powder. An accurately weighed quantity of Tablet powder equivalent to (50 mg of ATN & 12.5 mg of CTN) were transferred to 50.0 mL of volumetric flask and dissolved in sufficient amount of methanol. Then the volume was made up to the mark with methanol. The resultant solution was then filtered through whatman filter paper (no. 41). The filtered solution was then sonicated in ultrasonicator for 10.0 min. aliquot portions of 0.8 mL was then transferred to the three separate 10.0 mL volumetric flask and then the volume was mad up to the mark with mobile phase to get final concentration of (80.0 µg/mL of ATN and 20.0 µg/mL of CTN) respectively.

**Procedure:** Equal volume (20.0 µL) of standard and sample solution was injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured. The amount of drug in a Tablet was calculated using following formula

$$\text{mg/Tablet} = \frac{AT1 \times WS1 \times Ds \times P1}{AS1 \times WT \times Dt} \times \text{Avg. wt}$$

Where,

- AT1 = Average area of ATN/CTN peaks in Test chromatogram
- AS1 = Average area of ATN/CTN peaks in Standard chromatogram
- Ds = Dilution factor for standard

Dt = Dilution factor for test

P1 = Potency of working standards of ATN/CTN of % w/w basis

Avg. wt = Average weight of 10 Tablets

Further calculate the amount of ATN/CTN present in % of Label claim using following formula

$$\% \text{ Label Claim} = \frac{\text{Assay (mg/Tablet)} \times 100}{\text{Label claim of ATN/CTN}}$$

The results are shown in **Table 4**, while chromatogram is shown in **Figure 4**.

## 2.8 Method Validation

### 1. Linearity

#### Preparation of Standard Solutions

**ATN Standard Stock Solution (A)** Accurately weighed quantity of ATN (40.0 mg) was transferred to 10.0 mL volumetric flask and dissolved in methanol. The volume was made up to mark with methanol to get final concentration of (4000 µg/mL of ATN). The resultant solution was then sonicated for 10.0 min in ultrasonicator.

**CTN Standard Stock Solution (B)** Accurately weighed quantity of CTN (10.0 mg) was transferred to 10.0 mL volumetric flask and dissolved in methanol. The volume was made up to mark with methanol to get final concentration of (1000 µg/mL of CTN). The resultant solution was then sonicated for 10.0 min in ultrasonicator.

**Mixed Standard Solutions** aliquots portions of 0.1 to 0.5 mL from the standard stock solutions (A & B) were transferred to five 10.0 mL volumetric flasks and then volume was made up to the mark with mobile phase to get 5 different mixed standard solutions having concentrations of (40.0:10.0, 80.0:20.0, 120.0:30.0, 160.0:40.0, 200.0:50.0 µg/mL of ATN & CTN) respectively. The resultant solutions was then sonicated in ultrasonicator for 10.0 min

**Procedure** Equal volumes (20.0 µL) of 5 mixed standard solutions were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured. Then calibration curve (Peak area vs concentration) was plotted and it is shown in **Figure 5 & 6**. The observations are shown in **Table 5**.

### 2. Accuracy

**Preparation of Standard Solutions** Standard solutions of (ATN & CTN) were prepared at the level of 80 %, 10.00 %, 120 %.

**Preparation of Sample Solution** To the preanalysed sample solution (80 µg/mL of ATN & 20 µg/mL of CTN) a known amount of standard solutions of pure drugs (ATN & CTN) were added in different levels i.e. 80%, 10.00 %, 120%. The results of recovery studies shown in **Table 6**. The percent recovery was then calculated by using formula;

$$\% \text{ Recovery} = \frac{E_w - B}{C} \times 100$$

Where,

$E_w$  = Total drug estimated (mg)

B= Amount of drug contributed by pre analyzed Tablet powder (mg)

C= Weight of pure drug added (mg)

### 3. Precision

#### 3.1 Intra-Day Precision

It was determined by analyzing the 3 different solutions having concentration (80.0 µg/mL of ATN & 20.0 µg/mL of CTN) at 3 different times over a period of day.

#### 3.2 Inter-Day Precision

It was determined by analyzing the 3 different solutions having concentration (80.0 µg/mL of ATN & 20.0 µg/mL of CTN) at 3 days over a period of week.

**Procedure** Equal volumes (20.0 µL) of these solutions were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak areas, retention time of major peaks were measured. The results are shown in **Table 7 & 8**.

### 4. Specificity

Specificity is an ability to measures accurately and specifically the analyte of interest in the other components that may be expected to be present in the sample matrix.

**Preparation of Standard Solutions** The standard solutions were prepared as per the methodology adopted for laboratory mixtures.

**Preparation of Sample Solution:** Sample solution of marketed formulation was prepared as per the methodology adopted for marketed formulation analysis.

**Procedure:** Equal volume (20.0 µL) of standard and sample solution was injected separately after equilibrium of stationary

phase. The chromatograms were recorded and the response i.e. peak area, retention time of the major peaks were measured. Along with this the interference between the active ingredient and its excipients was also checked. The corresponding chromatograms are shown in **Figure 12 & 13**.

### 5. Robustness

**Preparation of Sample Solution:** Sample solution of marketed formulation was prepared as per the methodology adopted for marketed formulation analysis.

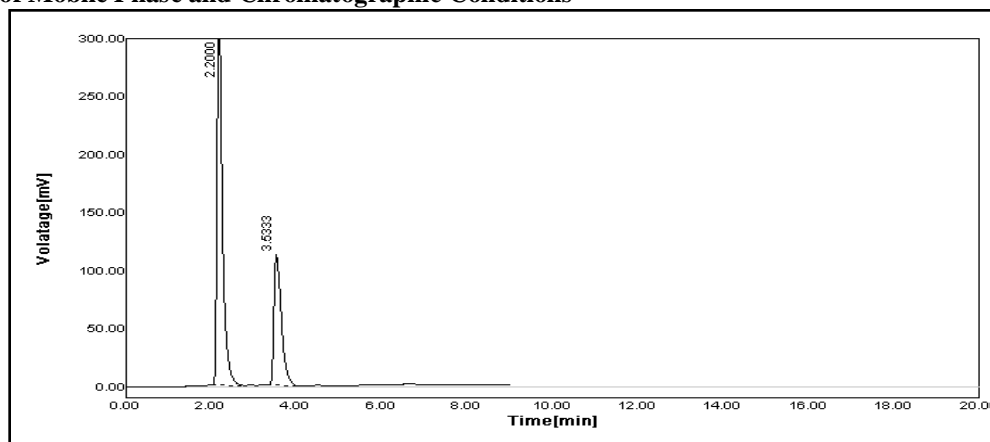
**Procedure:** Equal volume (20.0  $\mu\text{L}$ ) of sample solution was injected separately after equilibrium of stationary phase. Then deliberate variation in method parameters such as flow rate ( $<0.2\text{mL}/\text{min}$ ), change in detection wavelength ( $<2\text{ nm}$ ) was carried out. The chromatograms were recorded and the response i.e. peak area, retention time of the major peaks were measured. The results are shown in **Table 9** chromatograms are shown in **Figure 14 & 15**.

### 6. Ruggedness

Ruggedness of the method was studied by two different analysts using same operational and environmental conditions. A sample solutions prepared as per the methodology adopted in section 5.2 having concentration (80.0  $\mu\text{g}/\text{mL}$  of ATN & 20.0  $\mu\text{g}/\text{mL}$  of CTN) respectively, were analyzed and concentrations were determined. The results are shown in **Table 10**.

## 3. Results and Discussion

### Optimization of Mobile Phase and Chromatographic Conditions



**Figure 3 Optimized Chromatogram of ATN & CTN**

### Observation

Good resolution with minimized tailing also proper peak shape and system suitability was observed within the limits. Hence the above chromatographic parameters are finalized.

### System Suitability Studies

**Table 1 Result of System Suitability Studies for (ATN)**

System Suitability Test (ATN)					
Sr. No	Area Reproducibility	Retention Time	Tailing Factor	Resolution	Theoretical Plates
1	2840	2.3	1.71	0	3303
2	2853	2.3	1.71	0	3303
3	2856	2.31	1.71	0	3322
4	2854	2.31	1.71	0	3322
5	2860	2.31	1.72	0	3322
<b>Mean</b>	2852.6	2.306	1.712	0	3314.4
<b>SD</b>	7.536	0.0054	0.0044	0	10.40
<b>%RSD</b>	0.264	0.237	0.261	0	0.313
<b>Limit</b>	<b>NMT 2%</b>	<b>NMT 1%</b>	<b>&lt; 2</b>	<b>&gt; 2</b>	<b>&gt; 2000</b>

**Observation**

All the parameters of system suitability are observed within the limits for ATN.

**Table 2 Results of System Suitability Studies for (CTN)**

System Suitability Test (CTN)					
Sr. No	Area Reproducibility	Retention Time	Tailing Factor	Resolution	Theoretical plates
1	1366	3.51	1.75	3.31	2460
2	1376	3.51	1.75	3.31	2460
3	1379	3.51	1.81	3.27	2460
4	1363	3.51	1.75	3.31	2460
5	1375	3.5	1.75	3.27	2698
<b>Mean</b>	1371.8	3.508	1.762	3.294	2507.6
<b>SD</b>	6.90	0.0044	0.0268	0.021	106.43
<b>%RSD</b>	0.503	0.127	1.52	0.665	4.24
<b>Limit</b>	<b>NMT 2%</b>	<b>NMT 1%</b>	<b>&lt; 2</b>	<b>&gt; 2</b>	<b>&gt; 2000</b>

**Observation**

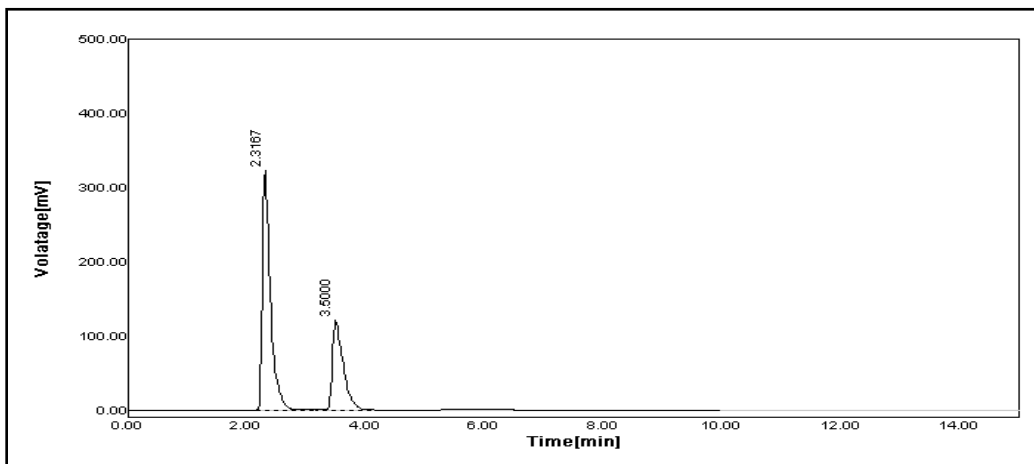
All the parameters of system suitability are observed within the limits for CTN.

**Analysis of Standard Laboratory Mixtures**

Analysis of Standard Laboratory Mixture											
Sr. No	Standard Amount Taken [ $\mu\text{g/mL}$ ]		Sample Amount Taken [ $\mu\text{g/mL}$ ]		Area of Standard		Area of Sample		% Amount Estimated		
	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	
1	40	10	40	10	2840	1366	2825	1361	99.47	99.63	
2	80	20	80	20	3840	1383	3827	1376	99.66	99.49	
					<b>Mean</b>	3340	1374.5	3326	1368.5	99.56	99.56
					<b>SD</b>	707.1	12.02	708.5	10.6	0.134	0.099
					<b>%RSD</b>	21.17	0.874	21.3	0.775	0.1346	0.0995

**Table 3 Results of Analysis of Standard Laboratory Mixtures****Analysis of Marketed Formulation****Table 4 Results of Marketed Formulation Analysis**

Analysis of Marketed Formulation												
Sr. No	Standard Amount Taken [ $\mu\text{g/mL}$ ]		Sample Amount Taken [ $\mu\text{g/mL}$ ]		Area of Standard		Area of Sample		Amount Found [ $\mu\text{g/mL}$ ]		% Amount Found	
	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN
1			80	20			3068	1510	79.9	19.9	99.875	99.5
2	80	20	80	20	3102	1540	3080	1521	80.5	20.3	100.62	101
3			80	20			3075	1517	80.2	20.1	100.25	100.5
					<b>Mean</b>		3074.3	1516	80.2	20.1	100.25	100.5
					<b>SD</b>		6.0	5.56	0.3	0.2	0.375	1
					<b>%RSD</b>		0.19	0.367	0.374	0.99	0.374	0.995

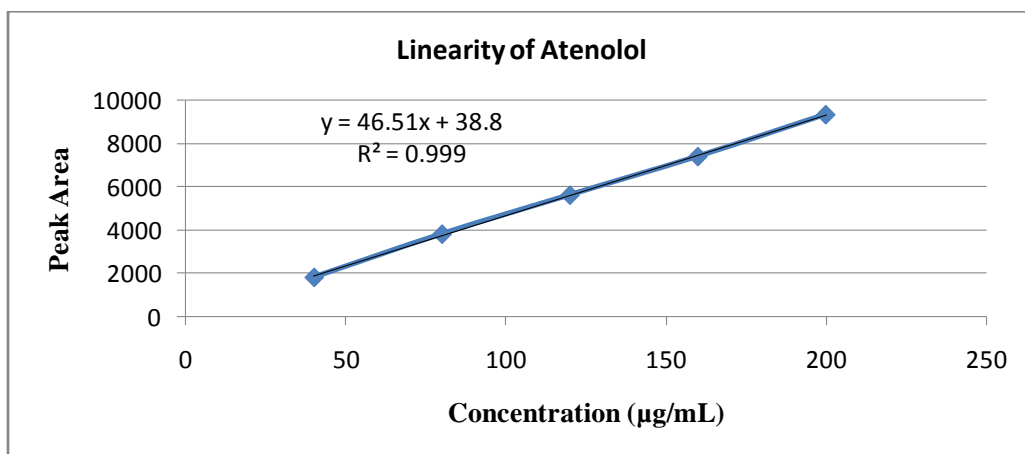


**Figure 4 Chromatogram of Marketed Formulation**

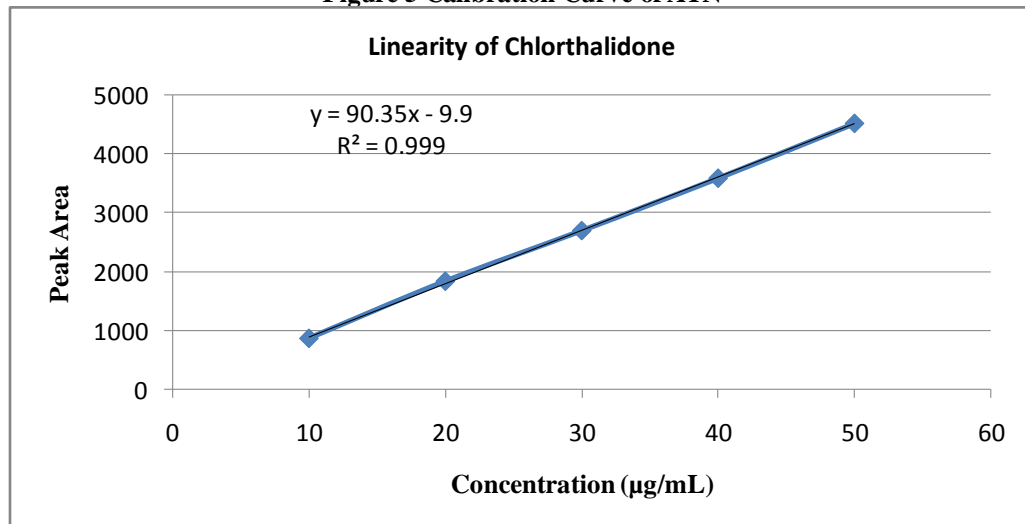
The proposed method was applied to the determination of ATN & CTN in marketed formulation the **mean % amount** found was **100.25 (ATN) & 100.5 (CTN)** with **% RSD** values is **NMT 2.0%** indicates the developed method was successfully applied for analysis of marketed formulation. All the results found are in good agreement with the label content of marketed formulation.

**Method Validation**

**1. Linearity**



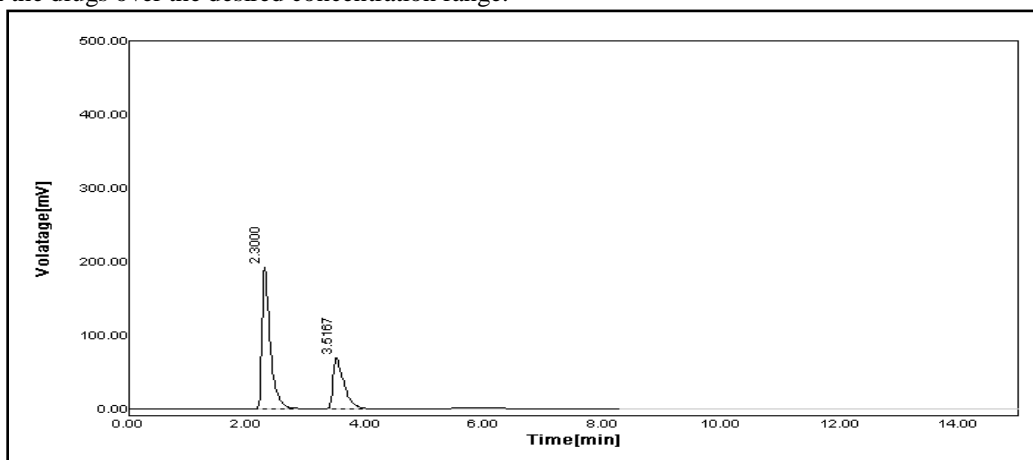
**Figure 5 Calibration Curve of ATN**



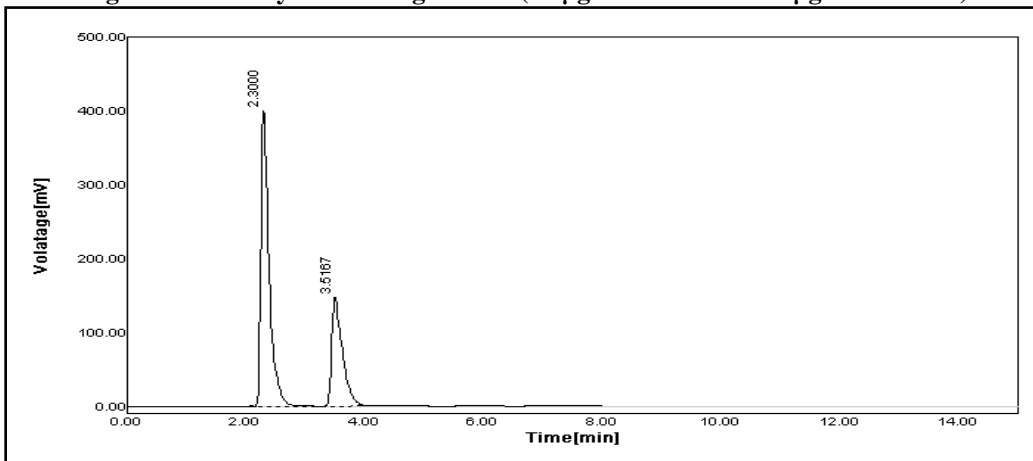
**Figure 6 Calibration Curve of CTN**  
**Table 5 Linearity Studies of ATN & CTN**

Concentration (µg/mL)		Peak Area	
ATN	CTN	ATN	CTN
40	10	1844	870
80	20	3840	1838
120	30	5640	2696
160	40	7420	3585
200	50	9356	4514
	<b>Mean</b>	5620	2700.6
	<b>SD</b>	2942.13	1428.79
	<b>%RSD</b>	52.35	52.90

In both calibration curves the  $r^2$  value was found to be **0.999** which nearly equals to unity. The regression equation for ATN was  $y = 46.51x + 38.8$  while for CTN it was  $y = 90.35x - 9.9$ . It indicates the capability of developed method to estimate both the drugs over the desired concentration range.

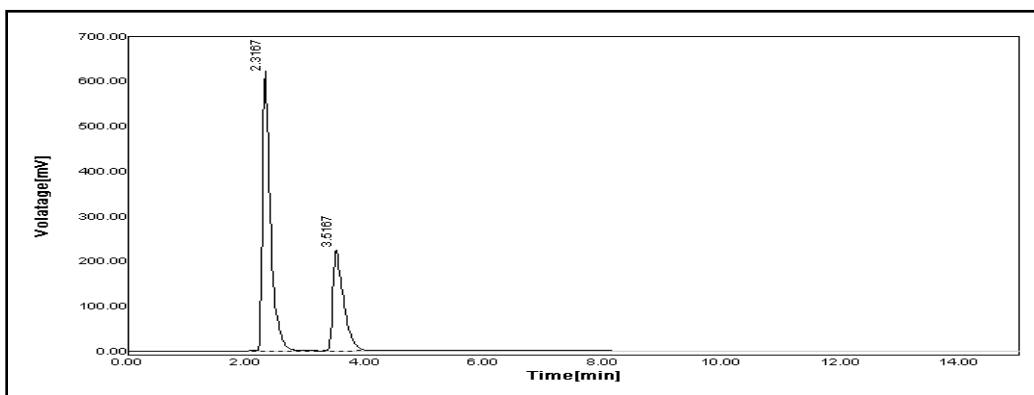


**Figure 7 Linearity Chromatogram for (40 µg/mL of ATN & 10 µg/mL of CTN)**

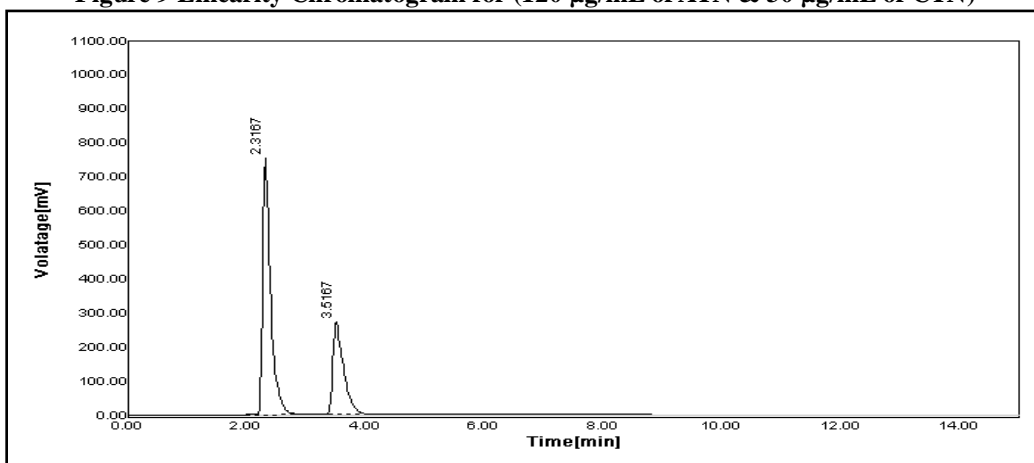


**Figure 8 Linearity Chromatogram for (80 µg/mL of ATN & 20 µg/mL of CTN)**

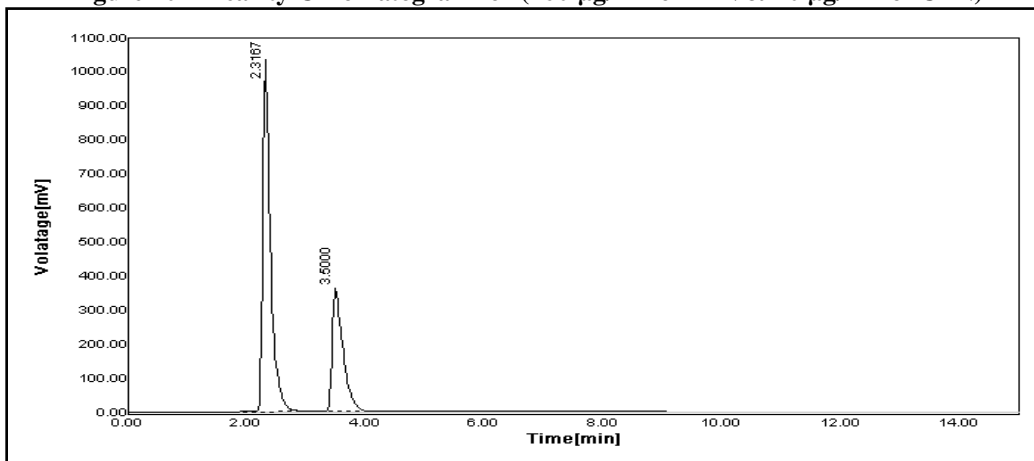




**Figure 9 Linearity Chromatogram for (120 µg/mL of ATN & 30 µg/mL of CTN)**



**Figure 10 Linearity Chromatogram for (160 µg/mL of ATN & 40 µg/mL of CTN)**



**Figure 11 Linearity Chromatogram for (200 µg/mL of ATN & 50 µg/mL of CTN)**

## 2. Accuracy

This is performed on the basis of recovery studies by standard addition method. Standard solutions of pure drugs (ATN & CTN) were added in different levels i.e. 80%, 100 %, 120%.

Table 6 Results of Recovery Studies

Recovery Studies										
Sr. No	Pre-analysed Sample Solution [ $\mu\text{g/mL}$ ]		Excess Pure Drug Added [ $\mu\text{g/mL}$ ]		Amount Recovered [ $\mu\text{g/mL}$ ]		Area		% Recovery	
	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN
1	80	20	64	16	63.8	15.8	6144	3231	99.68	98.75
2	80	20	80	20	79.9	20	7680	4038	99.87	100
3	80	20	96	24	95.8	23.8	9216	4845	99.79	99.16
								<b>Mean</b>	99.78	99.30
								<b>SD (n=3)</b>	0.093	0.636
								<b>% RSD (n=3)</b>	0.094	0.640

The mean % recovery with % RSD for ATN was found to be **99.78, 0.094** while for CTN it was **99.30, 0.640**. The % RSD here does not exceed 2 which fully agrees with system suitability hence the developed RP-HPLC method was found to be sufficiently accurate.

### 3. Precision

#### 3.1 Intra- Day Precision

Table 7 Results of Intra- Day Precision Studies

Sr. No	Samples	Amount Taken [ $\mu\text{g/mL}$ ]		Retention Time		Area of Peaks		Amount Found [ $\mu\text{g/mL}$ ]		% Amount Found		
		ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	
1	Precision 1 (I <sup>st</sup> Time)	80	20	2.3	3.5	3068	1510	79.9	19.9	99.8	99.5	
2	Precision 2 (II <sup>nd</sup> Time)	80	20	2.3	3.5	3072	1516	80.2	20.1	100.2	101	
3	Precision 3 (III <sup>rd</sup> Time)	80	20	2.31	3.5	3076	1517	80.5	20.3	100.6	102	
				<b>Mean</b>	2.30	3.5	3072	1514.3	80.2	20.1	100.2	101
				<b>SD</b>	0.005	0	4	3.78	0.3	0.2	0.375	1
				<b>%RSD</b>	0.25	0	0.13	0.25	0.37	0.99	0.37	1

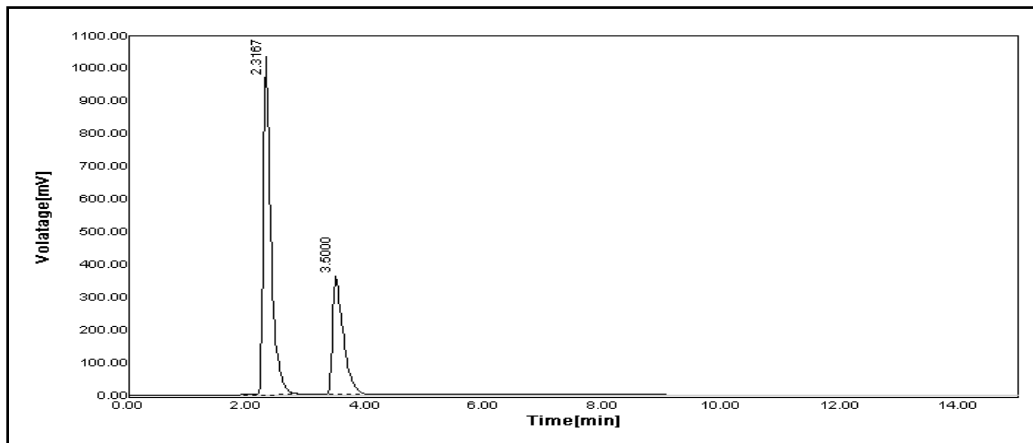
#### 3.2 Inter- Day Precision

Table 8 Results of Inter- Day Precision Studies

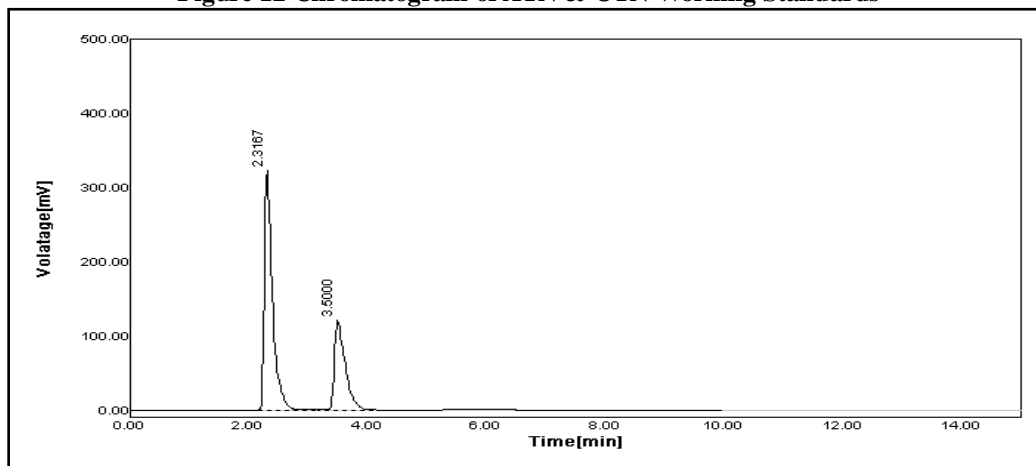
Sr. No	Samples	Amount Taken [ $\mu\text{g/mL}$ ]		Retention Time		Area of Peaks		Amount Found [ $\mu\text{g/mL}$ ]		% Amount Found		
		ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	
1	Precision 1 (Day 1)	80	20	2.3	3.51	3072	1510	80.2	19.9	100.25	99.5	
2	Precision 2 (Day 2)	80	20	2.31	3.5	3075	1508	80.35	19.8	100.43	99	
3	Precision 3 (Day 3)	80	20	2.3	3.51	3070	1512	80.3	20.2	100.37	101	
				<b>Mean</b>	2.30	3.50	3072	1510	80.2	19.9	100.3	99.8
				<b>SD</b>	0.005	0.006	2.51	2	0.076	0.20	0.09	1.04
				<b>%RSD</b>	0.25	0.16	0.08	0.132	0.095	1.04	0.095	1.042

Precision was determined as Intra-day & Inter-day precision. Reproducibility in retention time and peak area is seen in both intra and inter day precision studies with a %RSD (NMT than 2%) for both retention time and peak area which is in agreement with system suitability. Therefore, the proposed HPLC method for the determination of ATN and CTN in a tablet was found to be sufficiently precise.

**4. Specificity**



**Figure 12 Chromatogram of ATN & CTN Working Standards**



**Figure 13 Chromatogram of Marketed Formulation (Specificity)**

In the chromatogram obtained with working standard and marketed formulation solution interference is not observed at the retention time of any peak. Therefore, the proposed HPLC method for the determination of ATN and CTN in a tablet was found to be specific.

**5. Robustness**

**Table 9 Results of Robustness Studies**

Sr. No	Samples	Condition	Amount Taken [µg/mL]		Retention Time		Area of Peaks		% Amount Found		
			ATN	CTN	ATN	CTN	ATN	CTN	ATN	CTN	
1	Robustness 1	Flow rate (<0.2 mL/min)	80	20	2.31	3.5	7179	3737	99.7	99.5	
2	Robustness 2	Wavelength(<2.0 nm)	80	20	2.34	3.54	7236	3746	100.2	100.5	
					<b>Mean</b>	2.32	3.52	7207.5	3741.5	100	100
					<b>SD</b>	0.02	0.02	40.3	6.36	0.35	0.70
					<b>%RSD</b>	0.91	0.80	0.5	0.17	0.35	0.70

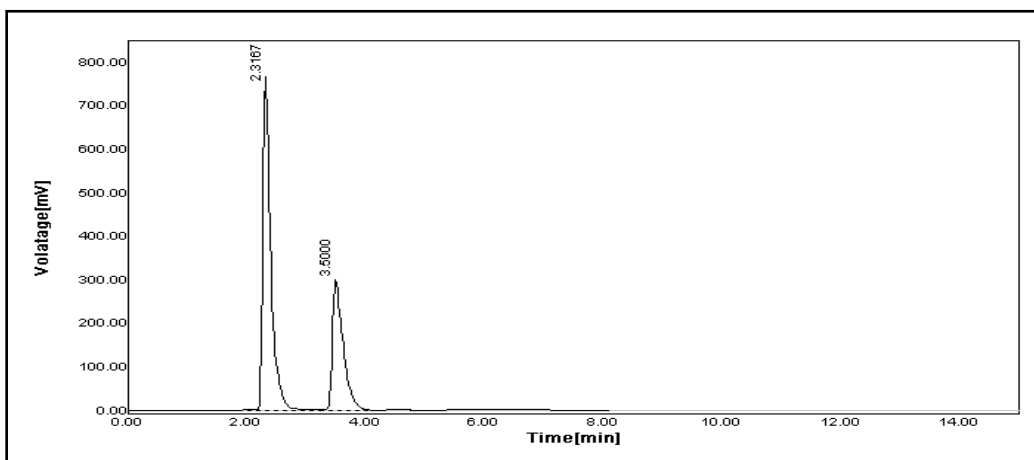


Figure 14 Chromatogram of Robustness (<2.0 nm)

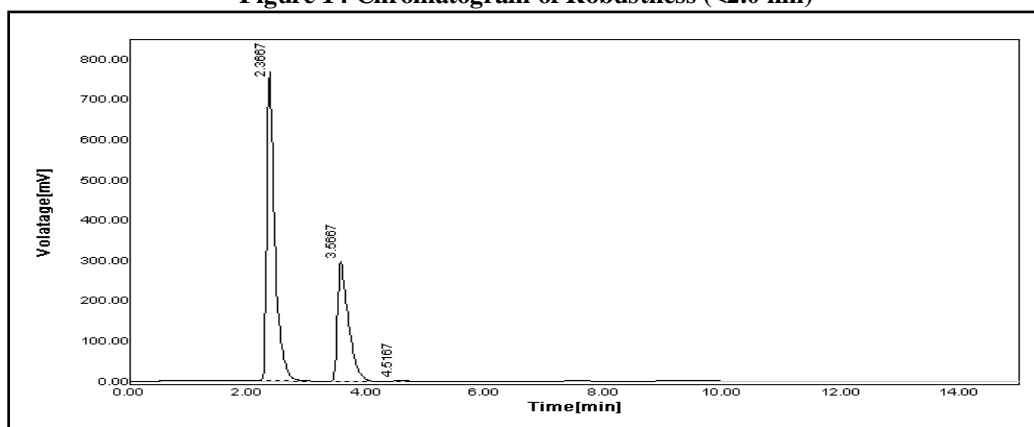


Figure 15 Chromatogram of Robustness (<0.2mL/min)

The results of assay of test solution were not affected by varying the conditions. They fully agree with the results obtained under original conditions. The % RSD for (Retention time, Peak area and % Amount Found) is not more than 2% for both (ATN & CTN) which is in agreement with system suitability. Hence the proposed HPLC method for the determination of ATN and CTN in a tablet was found to be **robust**.

6. Ruggedness

Table 10 Results of Ruggedness Studies

Sr. No	Amount Taken [µg/mL]		% Amount Found					
			Intra-Day		Inter-Day		Different Analyst	
	ATN	CTN	ATN	CTN	ATN	ATN	ATN	CTN
1	80	20	99.88	99.5	100.25	99.5	99.8	100
2	80	20	100.3	100.5	100.43	99	100.2	101
3	80	20	100.6	101.5	100.37	101	100.0	100.5
		<b>Mean</b>	100.3	100.5	100.35	99.8	100	100.5
		<b>SD</b>	0.375	1	0.09	1.04	0.265	0.70
		<b>%RSD</b>	0.374	0.9	0.095	1.042	0.56	0.72

The mean % amount found for (ATN & CTN) by different analyst was found to be 100% (ATN) and 100.5%(CTN) also the % RSD values for (Intra-Day, Inter-Day and Different analyst) studies for both the drugs is not more than 2% for both (ATN & CTN) which is agreement with system suitability hence the proposed HPLC method for the determination of ATN and CTN in a tablet was found to be **rugged**.

#### 4. Conclusion

The developed RP-HPLC method was found to be simple, accurate, sensitive, precise, specific, economical and rapid. The developed RP-HPLC method shows the good resolution between ATN and CTN within the run time of 20 min. The developed RP-HPLC method is very simple involving no complicated sample preparations. The developed RP-HPLC method was found to be highly specific. The developed RP-HPLC method was found to be linear over wider concentration range. Therefore the developed RP-HPLC method can be applied for routine quantitative and qualitative analysis of ATN and CTN in bulk and pharmaceutical formulations like tablets. The developed RP-HPLC method was validated as per the ICH guidelines.

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