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

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ATN, CTN, RP-HPLC, Assay method, Method Validation.

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. Analytical method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantify 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. From the literature review 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 and economical RP-HPLC method for determination of Atenolol (ATN) and Chlorthalidone (CTN) in combine dosage form.

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, 5µm) 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 µg/mL for ATN and 10-50 µg/mL for CTN. The calibration curves were linear (r² = 0.999 for ATN and r² = 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.

Figure 1 Chemical Structure of Atenolol

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2. Experimental

2.1 Reagents & Chemicals

Standard samples of ATN & CTN were received as gift samples from the Leben laboratories (Mumbai) and IPCA Laboratories (Mumbai). 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 of analytical grade.

2.2 Instruments

HPLC system of Younglin Quaternary pump with UV-VIS detector (190-990 nm) Software – Autocho. 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 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 is used 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 into 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 concentration of (40.0 µg/mL of ATN & 10.0 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 –

\[
\% \text{ Estimation} = \frac{At \times Ds \times Ws}{As \times Dt \times Wt} \times 100
\]

Where,

- \(At\) = Area count for sample solution
- \(As\) = Area count for standard solution
- \(Ds\) = Dilution factor for standard
- \(Dt\) = Dilution factor for sample
- \(Ws\) = Weight of standard (mg)
- \(Wt\) = 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. then aliquot portions of 0.8 mL was then transferred to the three separate 10.0 mL volumetric flask and then 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. 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 –

\[
\text{mg/Tablet} = \frac{AT1 \times WS1 \times Ds \times P1}{AT1 \times WS1 \times Ds \times P1} \times \text{Avg. wt}
\]

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,

Ew = 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 µL) of sample solution was injected separately after equilibrium of stationary phase. Then deliberate variation in method parameters such as flow rate (<0.2mL/min), change in detection wavelength (<2 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 µg/mL of ATN & 20.0 µg/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**

![Optimized Chromatogram of ATN & CTN](image)

**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>
<thead>
<tr>
<th>Sr. No</th>
<th>Area Reproducibility</th>
<th>Retention Time</th>
<th>Tailing Factor</th>
<th>Resolution</th>
<th>Theoretical Plates</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>2.3</td>
<td>1.71</td>
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<td>0</td>
<td>3322</td>
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<tr>
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<tr>
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<td>0.0044</td>
<td>0</td>
<td>10.40</td>
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<tr>
<td>% RSD</td>
<td>0.264</td>
<td>0.237</td>
<td>0.261</td>
<td>0</td>
<td>0.313</td>
</tr>
<tr>
<td>Limit</td>
<td>NMT 2 %</td>
<td>NMT 1 %</td>
<td>&lt; 2</td>
<td>&gt; 2</td>
<td>&gt; 2000</td>
</tr>
</tbody>
</table>
Observation
All the parameters of system suitability are observed within the limits for ATN.

Table 2 Results of System Suitability Studies for (CTN)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Area Reproducibility</th>
<th>Retention Time</th>
<th>Tailing Factor</th>
<th>Resolution</th>
<th>Theoretical plates</th>
</tr>
</thead>
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<td>1.75</td>
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<tr>
<td>2</td>
<td>1376</td>
<td>3.51</td>
<td>1.75</td>
<td>3.31</td>
<td>2460</td>
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<td>1.81</td>
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<tr>
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<td>1363</td>
<td>3.51</td>
<td>1.75</td>
<td>3.31</td>
<td>2460</td>
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<td>1.75</td>
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<td>SD</td>
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<td>0.0044</td>
<td>0.0268</td>
<td>0.021</td>
<td>106.43</td>
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<tr>
<td>%RSD</td>
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<td>0.127</td>
<td>1.52</td>
<td>0.665</td>
<td>4.24</td>
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<tr>
<td>Limit</td>
<td>NMT 2%</td>
<td>NMT 1%</td>
<td>&lt; 2</td>
<td>&gt; 2</td>
<td>&gt; 2000</td>
</tr>
</tbody>
</table>

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

Analysis of Standard Laboratory Mixtures

Table 3 Results of Analysis of Standard Laboratory Mixtures

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Standard Amount Taken [µg/mL]</th>
<th>Sample Amount Taken [µg/mL]</th>
<th>Area of Standard</th>
<th>Area of Sample</th>
<th>% Amount Estimated</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>ATN 40</td>
<td>ATN 40</td>
<td>2840</td>
<td>2825</td>
<td>99.47</td>
</tr>
<tr>
<td></td>
<td>CTN 10</td>
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<td>1361</td>
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<td>3326</td>
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<tr>
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<td></td>
<td>%RSD</td>
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<td>21.17</td>
<td>21.3</td>
<td>0.1346</td>
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</table>

Analysis of Marketed Formulation

Table 4 Results of Marketed Formulation Analysis

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Standard Amount Taken [µg/mL]</th>
<th>Sample Amount Taken [µg/mL]</th>
<th>Area of Standard</th>
<th>Area of Sample</th>
<th>Amount Found [µg/mL]</th>
<th>% Amount Found</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ATN 80</td>
<td>ATN 80</td>
<td>3068</td>
<td>3080</td>
<td>80.5</td>
<td>100.62</td>
</tr>
<tr>
<td></td>
<td>CTN 20</td>
<td>CTN 20</td>
<td>1510</td>
<td>1521</td>
<td>80.2</td>
<td>101.08</td>
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<td>3074.3</td>
<td>3102</td>
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<td>5.56</td>
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<td>0.375</td>
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<tr>
<td></td>
<td>%RSD</td>
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<td>0.19</td>
<td>0.367</td>
<td>0.374</td>
<td>0.374</td>
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</tbody>
</table>
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

Linearity of Atenolol

\[ y = 46.51x + 38.8 \]

\[ R^2 = 0.999 \]

Figure 5 Calibration Curve of ATN

Linearity of Chlorthalidone

\[ y = 90.35x - 9.9 \]

\[ R^2 = 0.999 \]
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.
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>
<thead>
<tr>
<th>Sr. No</th>
<th>Pre-analysed Sample Solution [µg/mL]</th>
<th>Excess Pure Drug Added [µg/mL]</th>
<th>Amount Recovered [µg/mL]</th>
<th>Area</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
</tr>
<tr>
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<td>20</td>
<td>64</td>
<td>16</td>
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<td>3</td>
<td>80</td>
<td>20</td>
<td>96</td>
<td>24</td>
<td>95.8</td>
</tr>
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</table>

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>
<thead>
<tr>
<th>Sr. No</th>
<th>Samples</th>
<th>Amount Taken [µg/mL]</th>
<th>Retention Time</th>
<th>Area of Peaks</th>
<th>Amount Found [µg/mL]</th>
<th>% Amount Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
</tr>
<tr>
<td>1</td>
<td>Precision 1 (I&lt;sup&gt;st&lt;/sup&gt; Time)</td>
<td>80</td>
<td>20</td>
<td>2.3</td>
<td>3.5</td>
<td>3068</td>
</tr>
<tr>
<td>2</td>
<td>Precision 2 (II&lt;sup&gt;nd&lt;/sup&gt; Time)</td>
<td>80</td>
<td>20</td>
<td>2.3</td>
<td>3.5</td>
<td>3072</td>
</tr>
<tr>
<td>3</td>
<td>Precision 3 (III&lt;sup&gt;rd&lt;/sup&gt; Time)</td>
<td>80</td>
<td>20</td>
<td>2.31</td>
<td>3.5</td>
<td>3076</td>
</tr>
</tbody>
</table>

Mean 2.30 3.5 3072 1514.3 80.2 20.1 100.2 101

SD (n=3) 0.005 0 4 3.78 0.3 0.2 0.375 1

% RSD 0.25 0 0.13 0.25 0.37 0.99 0.37 1

3.2 Inter-Day Precision

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Samples</th>
<th>Amount Taken [µg/mL]</th>
<th>Retention Time</th>
<th>Area of Peaks</th>
<th>Amount Found [µg/mL]</th>
<th>% Amount Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
</tr>
<tr>
<td>1</td>
<td>Precision 1 (Day 1)</td>
<td>80</td>
<td>20</td>
<td>2.3</td>
<td>3.51</td>
<td>3072</td>
</tr>
<tr>
<td>2</td>
<td>Precision 2 (Day 2)</td>
<td>80</td>
<td>20</td>
<td>2.31</td>
<td>3.5</td>
<td>3075</td>
</tr>
<tr>
<td>3</td>
<td>Precision 3 (Day 3)</td>
<td>80</td>
<td>20</td>
<td>2.3</td>
<td>3.51</td>
<td>3070</td>
</tr>
</tbody>
</table>

Mean 2.30 3.50 3072 1510 80.2 19.9 100.3 99.8

SD (n=3) 0.005 0.006 2.51 2 0.076 0.20 0.09 1.04

% RSD 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

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>
<thead>
<tr>
<th>Sr. No</th>
<th>Samples</th>
<th>Condition</th>
<th>Amount Taken [µg/mL]</th>
<th>Retention Time</th>
<th>Area of Peaks</th>
<th>% Amount Found</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
<td>CTN</td>
</tr>
<tr>
<td>1</td>
<td>Robustness 1</td>
<td>Flow rate (&lt;0.2 ml/min)</td>
<td>80</td>
<td>20</td>
<td>2.31</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>Robustness 2</td>
<td>Wavelength(&lt;2.0 nm)</td>
<td>80</td>
<td>20</td>
<td>2.34</td>
<td>3.54</td>
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<tr>
<td></td>
<td>Mean</td>
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<td></td>
<td></td>
<td>2.32</td>
<td>3.52</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td></td>
<td>%RSD</td>
<td></td>
<td></td>
<td></td>
<td>0.91</td>
<td>0.80</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Sr. No</th>
<th>Amount Taken [µg/mL]</th>
<th>Intra-Day</th>
<th>Inter-Day</th>
<th>Different Analyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATN</td>
<td>CTN</td>
<td>ATN</td>
<td>CTN</td>
</tr>
<tr>
<td>1</td>
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<td>20</td>
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<td>99.5</td>
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<tr>
<td>2</td>
<td>80</td>
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<td>100.3</td>
<td>100.5</td>
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<td>3</td>
<td>80</td>
<td>20</td>
<td>100.6</td>
<td>101.5</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Amount Found</td>
<td>Intra-Day</td>
<td>Inter-Day</td>
<td>Different Analyst</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%RSD</td>
<td>0.374</td>
<td>0.9</td>
<td>0.095</td>
<td>1.042</td>
<td>0.56</td>
<td>0.72</td>
<td></td>
</tr>
</tbody>
</table>

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.

References

5. Indian Pharmacopoeia, Government of India, Ministry of Health and Family Welfare. New Delhi; Published by the Controller of Publications; (2010), Vol.2, pp. 129.