A rapid, specific, sensitive High-performance liquid chromatographic method has been developed for determination of Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide impurities and its degradation products in pharmaceuticals preparation. 13 impurities including degradation as well as process related impurities have been well separated. HPLC was performed on a C18 column with “mobile phase A” consisting of Phosphate buffer pH 4.0; while “mobile phase B” consisted of 90:10v/v of Acetonitrile and water. The mobile phase was pumped in a gradient manner at the flow-rate of 1.2 mL min$^{-1}$. Ultraviolet detection was performed at 237 nm. Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide and its degradation products along with process impurities were chromatographed. Upon development, the method was evaluated by QbD approach, where 16 different experiments were carried out to evaluate the AQbD approach. Based on these experiments, the developed method was found meeting all the requirements of analytical QbD. Calibration showed that response of impurities was a linear function of concentration over the range LOQ to 150% of the target concentration ($r^2 \geq 0.999$) and the method was validated over this range for precision, accuracy, linearity and specificity. For precision study, percentage relative standard deviation of each impurity was $<15\%$ ($n = 6$). The method was found to be precise, accurate, linear and specific. The method was successfully employed for estimation of Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide impurities and its degradation products in finished product Tablets formulation.

**Keywords:**
HPLC – Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide, Impurities and Method validation

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**Corresponding Author:** Avinash S. Patil, Department of Chemistry, Jawaharlal Nehru Technological University, Hyderabad, India.
INTRODUCTION:
Hypertension is the "silent killer" of humans because this disease is usually asymptomatic until the damaging effects of hypertension such as coronary heart disease and stroke. Amlodipine besylate is chemically described as (3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate benzene sulphonate). Amlodipine besylate is a calcium-channel blocking agent; a dihydropyridine derivative with an intrinsically long duration of action. Amlodipine besylate is an anti-hypertensive and an antianginal agent in the form of the besylate salt [1-3]. Candesartan Cilexetil is chemically described as (±)-1-Hydroxyethyl 2-ethoxy-1-[p-(o-1H-tetrazol-5ylphenyl)benzyl]-7-benzimidazolecarboxylate, cyclohexyl carbonate (ester). Candesartan Cilexetil is a prodrug, is hydrolyzed to candesartan during absorption from the gastrointestinal tract. Candesartan is a selective AT1 subtype angiotensin II receptor antagonist. Candesartan cilexetil (candesartan) is a drug used for treating high blood pressure (hypertension). Hydrochlorothiazide is chemically described as (6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide).

Hydrochlorothiazide

Figure-1: Chemical structures

Candesartan Cilexetil and Hydrochlorothiazide, Candesartan Cilexetil and Amlodipine drugs are official in the British Pharmacopoeia, Indian Pharmacopoeia and United States Pharmacopoeia but combination of these three drugs is not official. Based on the literature survey, no official method has yet
been developed for their separation and its impurities [6-8]. Several methods have been reported using HPLC with UV and fluorescent detection for the determination of Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide individually in pharmaceutical dosage forms as well as in biological fluids [9-17]. Thus, application of an HPLC method with high sensitivity and selectivity will find use for the determination of Candesartan Cilexetil, Amlodipine, and hydrochlorothiazide impurities and its degradation products in pharmaceutical formulations.

**EXPERIMENTAL METHODS**

**Chemicals and Reagents**

Candesartan Cilexetil and its impurities, Candesartan ethyl ester, Ketone Cilexetil, Impurity-B, Impurity-A, N1-Ethyl Impurity, N-Cilexetil ethyl ester and N-Ethyl Candesartan Cilexetil, and Hydrochlothiazide and its impurities, Chlorothizide, Benzothiazide and 5-Chlorothizide impurities from USP and Amlodipine and its impurities, Amlodipine-Methyl ester, Amlodipine-Ethyl ester, Amlodipine Impurity A, Amlodipine Impurity B and Amlodipine Impurity D. Acetonitrile (HPLC-grade from J.T. Baker, USA) was from Merck (Darmstadt, Germany). Water was purified by a Millipore (Bedford, MA, USA) Milli-Q water-purification system and passed through a 0.22 µm membrane filter (Durapore; Millipore, Dublin, Ireland) before use.

**Equipment**

HPLC analysis was performed with a Waters Alliance system equipped with a quaternary solvent manager, sample manager, column-heating compartment, and Photodiode array detector. This system was controlled by Waters Empower software. The specificity study was conducted by using heating oven, stability chamber and heating mantel (Thermo Lab, India).

**Standard and Sample Preparation**

The standard stock solution of Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide was prepared by dissolving an accurately weighed amount of working standards in diluent (Mix 700 volumes of Water with 300 volumes of Acetonitrile), resulting in a concentration of 0.4mg/mL, 0.36mg/mL and 0.62mg/mL respectively. Above solution further diluted in diluent to get a concentration of 4.0 µg mL⁻¹, 3.6 µg mL⁻¹ and 6.2 µg mL⁻¹ respectively. The impurity stock solutions for Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide impurities was prepared by dissolving an accurately weighed amount in diluent, resulting in a concentration of 4.0 µg mL⁻¹ of each impurity of Candesartan Cilexetil impurities, 3.6 µg mL⁻¹ of each impurity of Amlodipine impurities and 6.2 µg mL⁻¹ for Hydrochlorothiazide impurities. The test solution was prepared by dissolving an accurately weighed portion of the powder, equivalent to 80mg of Candesartan Cilexetil (50mg of Amlodipine and 125 mg of Hydrochlorothiazide) in 70mL diluent. After sonicating for around 30minutes, volume made up to 100mL. Above solution was filtered through 0.22 µ PVDF filter to eliminate insoluble excipients. The clear liquid used for chromatographic analysis.

**Chromatography**

The analytes were separated on an Waters HPLC with ACE C18 column (250mm x 4.6 mm, 5µ) at column oven temperature of 25°C with a gradient run program at a flow-rate of 1.2 mL min⁻¹ (Table 1). Before use, the mobile phase was filtered through a 0.22 µm Millipore filter. UV detection was performed at 237 nm. The sample injection volume was 10 µL.
Table 1. Gradient program for elution of Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide and impurities

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
<th>Elution</th>
</tr>
</thead>
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<tr>
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<td>90 ➔ 90</td>
<td>10 ➔ 10</td>
<td>linear gradient</td>
</tr>
<tr>
<td>2–5</td>
<td>90 ➔ 85</td>
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</tr>
<tr>
<td>5–10</td>
<td>85 ➔ 70</td>
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<td>linear gradient</td>
</tr>
<tr>
<td>10–60</td>
<td>70 ➔ 20</td>
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<tr>
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</tr>
<tr>
<td>90–100</td>
<td>90 ➔ 10</td>
<td></td>
<td>re-equilibration</td>
</tr>
</tbody>
</table>

Table 2. Chromatographic Conditions

- LC Column: ACE C18 column (250 mm x 4.6 mm, 5 µ)
- Flow Rate: 1.2 mL/minutes
- Run Time: 100 minutes
- Wavelength: 237nm
- Column Temperature: 25°C

QbD Method Approach
Upon successfully development of HPLC method, further it has been evaluated by QbD approach. To check the developed method is enough robust, Selective, Specific i.e Analytical Target Profile (ATP) was achieved successfully or not.

Quality Attributes (QA) has been evaluated to sustain the method’s predefined ATPs, the responses for the peak of Candesartan, Amlodipine and Hydrochlorothiazide and its impurities are:
1. Spectral peak purity
2. Resolution from closely eluting peak
3. Theoretical plates (USP plate count)
4. Tailing factor (USP tailing)

The method parameters, accountable for an undesired variation in QAs consider as PLMPs, these are controllable and uncontrollable. The parameters possibly influencing any peak’s characteristics are as follows.

Controllable parameters:
1. Mobile phase pH
2. Acetonitrile ratio in mobile phase
3. Mobile phase flow
4. Column Temperature

Uncontrollable parameters:
Intermediate system performance (system to system, column to column, day to day, analyst to analyst, and mobile phase preparation’s variation).

Table 3. Full factorial experimental design table depicting blocking of uncontrollable factor and simultaneously varied controllable factors

<table>
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<th>pH</th>
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<th>Peak Purity of analytes</th>
<th>USP Tailing</th>
<th>Resolution</th>
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Design of Experiments (DoE)

To eliminate any system biasness, sixteen runs of uncontrollable parameters were executed by spiking 13 impurities at each sample preparation during the DoE trials to ensure optimum separation. Table 3 displays the compiled data for the obtained resolution value alongside simultaneously varied factors under different blocks. This also revealed the relationship between CQA and predefined liable process parameters (PLMPs) and suggested performance of the statistical analysis of obtained data for resolution. “Half Normal plot” displays the absolute standard effect for all the considered PLMPs and their interactions. The Pareto chart revealed that the most influential term for the resolution is column temperature followed by the interaction term of mobile phase-pH and the Acetonitrile volume ratio in the Mobile phase B.
Fig. 2. Pareto Chart for the response.

Fig. 3. Contour plots as design space for the response against factors (a) Candesertan Impurity and Amlodipine and (b) Methyl Ester Impurity and Candesertan Impurity.
Method Validation

The method was validated for specificity, precision, accuracy, sensitivity and linear range as per the International Conference on Harmonization (ICH) guidelines [19].

Specificity:

A study was conducted to demonstrate the interference from placebo. Sample solutions were prepared by taking the placebo equivalent to the amount present in the sample solution and analyzed as per test method. Chromatograms of placebo preparations are not showing any interference at the retention time of known impurities as well as analyte peaks.

A study was conducted to demonstrate the known impurities interference by spiking the sample solution with all the known impurities at 0.5% spike level and analyzed as per test method. It is found that all the known impurities are separated from each other and also from analyte peaks.

The known impurities of Candesartan Cilexetil, Amlodipine, and hydrochlorothiazide were injected individually to confirm the retention time.

A study was conducted to demonstrate the effective separation of degradants from Candesartan Cilexetil, Amlodipine, and hydrochlorothiazide peaks. The drug product was subjected to hydrolysis by refluxing the test solution in 1 N Sodium hydroxide solution at 55ºC for 15 minutes. Similarly the acidic hydrolysis was performed by refluxing test solution in 1N Hydrochloric acid solution at 55ºC for 15 minutes. The neutral hydrolysis was done in water at refluxing temperature of 55ºC for 15 minutes. Oxidation studies were performed in 10 % Hydrogen Peroxide solution at Bench top for 15 minutes. On photo stability study drug product was sufficiently spread on petri plates (1 mm thick layer) and exposed to sunlight and UV light at ambient conditions for 5 hours. Thermal degradation study was performed by heating drug product at 85º C for 12 hours.

Stressed samples were injected into the HPLC system with photo diode array detector by following test method conditions.

Precision:

The precision of test method was evaluated by using six samples spiked with known Impurities at 0.5% level and analyzed as per test method.

Accuracy:

To confirm the accuracy of the method, recovery studies were carried out by standard addition technique. Samples were prepared in triplicate by spiking all known impurities in test preparation at the level of LOQ, 50%, 100% and 150% of the standard concentration and analyzed as per the test method.

Sensitivity:

Sensitivity of the method was established with respect to Limit of detection and limit of quantification for Candesartan Cilexetil, Amlodipine, and hydrochlorothiazide impurities. Series of concentration of drug solution and its impurities were injected, LOD and LOQ established by Signal to Noise ratio method.

Precision was performed at LOQ level for all the known impurities by injecting six replicate injection of each impurity at the concentration obtained from above method.

Linearity of Detector Response:

A series of solutions of all the known impurities in the concentration ranging from limit of quantification level LOQ to 150% of standard concentration were prepared and injected into the HPLC system.

RESULTS AND DISCUSSION

Selectivity, sensitivity, resolution, and speed of chromatographic separation were optimized for the HPLC method. The retention times of Candesartan Cilexetil at about 52.927 and it impurities – Candesartan Impurity A at about 56.964, Candesartan Impurity B at about 51.101, Candesartan Impurity Ketone at about 43.970, Candesartan Impurity N-Cilexetil at about 63.522, Candesartan Impurity N-Ethyl at about 69.853, Candesartan Impurity N-1-Ethyl at about 62.154. The retention time of Amlodipine at about 23.920 and its impurities--impurity - A at 54.150, impurity-D at
20.484, Amlodipine Methyl Ester at about 21.514 and Amlodipine Ethyl Ester at about 27.445. The retention time of Hydrochlorothiazide at about 10.259 and its impurities --Benzochlorothiazide at about 8.299, Chlorothiazide at about 9.388, under the chromatographic conditions described, and the total run time was 100 minutes. Chromatograms obtained from blank, diluted standard, controlled sample and Test sample spiked with all impurities are shown in Figures 4A, 4B, 4C, 4D and 4E respectively.
HPLC system has been proved to be a promising tool for separation. Use of ACE C18 column (250 mm x 4.6 mm, 5µ) as stationary phase enabled optimization of HPLC for both peak selectivity and separation of all thirteen impurities. Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide and its impurities were well separated with good peak shape and resolution. No interfering peaks were observed in blank & placebo, indicating that signal suppression or enhancement by the product matrices was negligible.

After satisfactory development of method and successfully evaluation by AQbD approach, it was subjected to method validation as per ICH guideline [19]. The method was validated to demonstrate that it is suitable for its intended purpose by standard procedure to evaluate adequate validation characteristics. The result of system suitability parameter was found to be complying acceptable suitability criteria: relative standard deviation of replicate injection is not more than 5.0%. The result of specificity study ascertained the known impurities are separated from each other and also from Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide peak and spectral purity of all exposed samples found spectrally pure. The % RSD of replicate determination was found to be <5 during precision study, which indicates that the method is precise. The result obtained in the recovery study were found within the range of 85% to 115% (LOQ to 150%), which indicates that method is accurate. Sensitivity of method was verified and method is found to be linear, accurate and precise at limit of quantification. The calibration curve of all impurities were obtained by plotting the peak area of individual impurity versus concentration over the range of LOQ to 150% and were found to be linear (r = 0.999). The applicability of the method was verified by the determination of impurities in In house formulation. The impurity content was found to be satisfactory in the finished product formulations.
CONCLUSION
A rapid, specific, sensitive High-performance liquid chromatographic method has been developed for determination of Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide and its degradation products in pharmaceuticals preparation. The developed method has been evaluated by QbD principle. A number of analytical approaches have been previously described to Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide individually in pharmaceutical dosage forms as well as in biological; however, this is the first study reporting a validated reversed phase method for impurity quantification in Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide formulation. Separately Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide drugs are official in the British Pharmacopoeia and United States Pharmacopoeia but their combination is not official. Based on the literature survey, no official method has yet been developed for their separation and its impurities. The simple HPLC method developed in this study makes it suitable for separation and estimation of impurities without interference from excipients and other related substances present in the pharmaceutical matrices. The analytical performance and the result obtained from analysis of the formulation demonstrated that the method is reliable and sufficiently robust. In conclusion, the high sensitivity, good selectivity, accuracy and reproducibility of the HPLC method developed in this study makes it suitable for quality control analysis of complex pharmaceutical preparation containing Candesartan Cilexetil, Amlodipine and Hydrochlorothiazide and its impurities.

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