INTRODUCTION
Amlodipine besylate (AML) (Figure 1a) is 3-Ethyl 5-methyl (4RS)-2-(2-aminoethoxy) methyl 4-(2-chlorophenyl)-6-di-hydropyrnidine-3,5-dicarboxylate benzene sulphonate which is a calcium channel blocker and widely used in the treatment of hypertension13-14. Olmesartan medoxamil (OLM) (Figure 1b) is (2,3-dihydroxy-2-butenyl) 4-(1-hydroxy-1-methyleryl)-2-propyl-1-(p-oH-tetrazol-5-ylphenyl) benzyl imidazole-5-carboxylate, cyclic 2,3-carbonate] is an angiotensin II receptor blocker (ARB)19. Olmesartan blocks the vasoconstrictor effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor in vascular smooth muscle and Hydrochlorothiazide (HCZ) (Figure 1c) is 6-chloro-3, 4-dihydro-2H-1,2,4-benzo thiadiazine-7-Sulphonamide 1,1-dioxide which is diuretic and antihypertensive drug12,26,30 that inhibits the re-absorption of sodium and calcium at the beginning of distal convoluted tubules. A combination of antihypertensive agents can better control blood pressure and reduce the number and severity of side effects than a mono therapy. Both angiotensin II type 1 receptor blockers and calcium channel blockers were shown to be efficacious in reducing cardiovascular risk. Telmisartan and amlodipine fixed dose combinations have been demonstrated in numerous clinical trials to be highly effective in lowering blood pressure and suggest that the combined use might be more effective in treating hypertension than a monotherapy5,6. As per the literature survey, AML is official in IP2, USP3 and BP4. Several analytical methods that have been reported for the estimation of AML in biological fluids and/or pharmaceutical formulations include UV spectrophotometric8,12, UV method in combination with other anti hypertensive drug and diuretics15,22 and HPLC method23,29, stability indicating HPLC method25,33, LC- MS/MS23,34, LC-ESI-MS/MS36, UPLC-electrospray ionization mass spectrometry27, thin layer chromatography method (TLC) and high performance thin layer chromatography (HPTLC) method38-41. Some methods have been reported with combination with OLM42-46, metoprolol47, nebivolol hydrochloride48,49 and HCZ50,51. Analytical methods for the estimation of OLM in bulk drug and their formulation include UV method2,3,5, HPLC2,4,5, stability indicating HPLC5,6,7 and some of the methods that have been for HCZ58-63. HCZ is official in IP2 and USP3. Analytical methods that have been reported for the estimation of HCZ in bulk drug and their formulation include UV spectrophotometric and HPLC as single or in combination of AML50,51, OLM58-63, nebivolol hydrochloride64,66, Eprosartan66 and Telmasartan67 and UV method has been reported for estimation of all three drugs68. However, no RP-HPLC method has yet been reported for simultaneous estimation of AML, OLM and HCZ in tablet dosage forms. Hence, an attempt has been made to develop and validate in accordance with ICH guidelines69.

MATERIALS AND METHODS
Instrument
Liquid chromatographic system from Young Lin 9100 comprising of manual injector, YL 9111 quaternary pump for constant flow and constant pressure delivery and Photodiode array detector (YL 9160 detector) connected to software YL clarity for controlling the instrumentation as well as processing the data generated was used.
Chemicals

Drugs
Pharmaceutically pure sample of AML was obtained from Sun pharmaceuticals, Silvasa (GJ), OLM was obtained from Plathico Pharma Ltd. Indore, India and HCZ was obtained from Matrix Laboratory Mumbai as gift samples along with their analytical reports. Commercial tablet of amlodipine besylate (5 mg), olmesartan medoxamil (20 mg) and Hydrochlorothiazide (12.5 mg), Olmat-AMH (Micro labs) were procured from the local drug market.

Solvent
Acetonitrile (HPLC Grade), Methanol (HPLC Grade), Potassium di hydrogen phosphates (AR grade), Disodium hydrogen phosphate (AR grade) was obtained from Merck chemical division, Mumbai and Milli-Q was used to prepare water used in RP-HPLC method.

Diluents
A mixture of acetonitrile: methanol: phosphate buffer pH-3.0 (48:12:40 % v/v/v) was used in RP-HPLC as diluents.

Selection of mobile phase
Initially to estimate AML, OLM and HCZ simultaneously, number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was acetonitrile: methanol: phosphate buffer pH-3 (48:12:40 % v/v/v), run as isocratic system. The mobile phase was filtered through 0.45 µ filter paper and then degassed by sonication. Flow rate employed for analysis was 1.2 ml/min.

Preparation of Stock Solution
Accurately weighed 100 mg of AML, OLM and HCZ were transferred into 100 ml volumetric flasks separately and dissolved in 50 ml of diluent, then volume was made up to 100 ml with diluent to get a concentration of 1000 µg/ml (Stock-A) for all three drugs.

Preparation of Sub Stock Solution
5 ml of solution was taken from stock-A of AML, OLM and HCZ and transferred into 50 ml volumetric flask separately and diluted up to 50 ml with diluent to give concentration of 100 µg/ml (Stock-B). From stock-B a series of dilution was made in the range of 5-25 µg/ml, for all three drugs.

System suitability parameters
Separation variable was set and mobile phase was allowed to saturate the column at 1.2 ml/min. After complete saturation of column, six replicates of reference standard, 15 µg/ml of AML, OLM and HCZ were injected separately. Peak report and column performance report were recorded at 232 nm for all chromatogram.

Preparation of calibration curve standards and quality control samples
To establish the linearity of analytical method, a series of dilution ranging from 5-25 µg/ml of AML, OLM and HCZ was prepared in the same manner as described above. All the solution were filtered through 0.2 µm membrane filter and injected, chromatograms were recorded at 232 nm and it was repeated for five times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

Stability study
Samples prepared for repeatability study were preserved for 24 h at room temperature and analyzed on the following day to test for short-term stability.

Preparation for analysis of tablet formulation
Twenty tablets were taken and their average weight was determined. They are crushed to fine powder; amount equivalent to 5 mg of amlodipine was taken in 100-ml volumetric flask. The olmesartan and hydrochlorothiazide present in this amount of tablet powder was 20 mg and 12.5 mg, the ratio of all three drugs were 5:20:12.5. This was then dissolve in 50 ml of methanol by sonication for about 10 minutes. The volume was made up to the mark by methanol and filtered by Whatmann filter paper (no. 41) and the filtrate was used to prepare samples of different concentration. Now all the tablet samples was scanned in multi photometric mode and the concentration of all three drugs were obtained from the equation.

Validation of Method
As per ICH guideline the method was validated and following parameters were evaluated.

Linearity
Linearity of AML, OLM and HCZ was established by response ratios of drug. The response ratios (response factor) were calculated by dividing the AUC with respective concentration. The curve was plotted between response ratios and concentration which shows the good linearity of drugs in the concentration ranging from 5-25 µg/ml for AML, OLM and HCZ respectively.

Specificity
Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components.

Precision
Precision was determined by repeatability, Intermediate precision and reproducibility of all three drugs.

Repeatability
The repeatability was performed for five replicate at five concentrations in linearity range 5, 10, 15, 20 and 25 µg/ml for AML, OLM and HCZ that indicates the precision under the same operating condition over short interval time.
Intermediate Precision
Day to day precision
Intermediate precision was also performed within laboratory variation on different days for all three drugs simultaneously in five replicate at five concentrations.

Analyst- to- analyst precision
Analyst to analyst variation was performed by different analyst in five replicate at five concentrations.

Reproducibility
The reproducibility was performed by chemical to chemical (use of Rankem chemicals in place of Merck chemicals) variation in five replicate at five concentrations.

Accuracy (% recovery)
This study was carried out using pre analyzed tablet solution. A definite concentration of pure drug was added (80 %, 100 % and 120 % level) and then recovery was studied. A pre analyzed tablet solution containing 5 µg/ml of AML 20 µg/ml of OLM and 12.5 µg/ml of HCZ were taken in 10 ml volumetric flasks and known concentrations of pure drug solution was added to them, which were prepared from standard stock solution of amlodipine, olmesartan and hydrochlorothiazide. It was repeated at 5 concentration and 3 replicate level. Calculation was done from the label claim and the average weight of the final product.

Robustness
As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check the method’s capacity to remain unaffected. The ratio of mobile phase was change from, ACN: Methanol: Phosphate buffer pH-3 (48:12:40 % V/V/V), to (55: 10:35 % V/V/V).

LOD and LOQ
The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

RESULTS AND DISCUSSION
Method development
The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of AML, OLM and HCZ from tablet dosage form. Initially to estimate AML, OLM and HCZ, simultaneously number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter (Table 1) like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was acetonitrile: methanol: phosphate buffer pH-3 (48:12:40 % v/v/v), run as isocratic system. The mobile phase was filtered through 0.45 µ filter paper and then degassed by sonication. Flow rate employed for analysis was 1.2 ml/min. Separation variable (Table 2) was set and mobile phase was allowed to saturate the column at 1.2 ml/min. After complete saturation of column, six replicates of reference standard, 15 µg/ml of AML, OLM and HCZ were injected separately. Peak report and column performance report were recorded. The chromatogram was recorded at 232 nm Figure 2a, 2b and 2c. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph Figure 3, Figure 4 and Figure 5. The result of their optical characteristics and linearity data of all three drugs has been reported in the Table 3.

Method Validation
Linearity
The proposed method was found to be linear in the range of 5-25 µg/ml for all three drugs with correlation coefficient 0.9997, 0.9998, and 0.9998 for AML, OLM and HCZ respectively. Linearity of AML, OLM and HCZ were established by response ratios of drug. Response ratio of three drugs was calculated by dividing the absorbance or peak area with respective concentration (Table 4).

Specificity
Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as matrix components. The result of specificity is shown in Figure 6 and Figure 7 as compare to blank, there was no interference seen in chromatogram.

Precision
Precision of the methods was studied at three levels as at repeatability, intermediate precision (Day to Day and analyst to analyst) and reproducibility (Table 5).

Accuracy
The validity and reliability of proposed methods were assessed by recovery studies. The recovery of added standards (80 %, 100 % and 120 %) was found at five replicate and five concentrations level. The values of % mean just close to 100, SD and % RSD were less than 2 which indicate the accuracy of method. Result of recovery study is shown in Table 6.

Robustness
The robustness of developed method was checked by changing in the deliberate variation in solvent. Result of robustness is shown in Table 7.

LOD and LOQ
Detection limit and Quantitation limit of described method were observed as 0.553 µg/ml and 1.676 µg/ml for AML, 0.546 µg/ml and 1.655 µg/ml for OLM, 0.474 µg/ml and 1.438 µg/ml for HCZ, based on the SD of response and slope, which meet the requirement of new method.

Assay of tablet formulation
The results of the analysis of tablet formulation (olmat-AMH) were reported. The assay value of AML, OLM and HCZ were close to 100, SD and % RSD are less than 2 which indicate that the no interference of excipient in the estimation of AML, OLM and HCZ was observed.
statistical evaluation of tablet analysis by methods has been reported in Table 8.

Table 1: Results of system suitability parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AML</th>
<th>OLM</th>
<th>HCZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>4.06 ± 0.009</td>
<td>4.93 ± 0.01</td>
<td>2.61 ± 0.01</td>
</tr>
<tr>
<td>Number of Theoretical plates</td>
<td>5101 ± 338.01</td>
<td>5100 ± 552.2</td>
<td>4291 ± 111.8</td>
</tr>
<tr>
<td>HETP</td>
<td>0.047 ± 0.003</td>
<td>0.049 ± 0.006</td>
<td>0.058 ± 0.001</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.60 ± 0.02</td>
<td>1.24 ± 0.017</td>
<td>1.75 ± 0.03</td>
</tr>
<tr>
<td>Resolution</td>
<td>1.45 ± 0.32</td>
<td>1.11 ± 0.11</td>
<td>2.61 ± 0.17</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>1.32</td>
<td>1.23</td>
<td>1.62</td>
</tr>
</tbody>
</table>

Table 2: Separation variable of RP-HPLC method

<table>
<thead>
<tr>
<th>Variable</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>250 mm x 4.60 mm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>5 µ</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Octadecyilsilane (C₁₈)</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>48 %</td>
</tr>
<tr>
<td>Methanol</td>
<td>12 %</td>
</tr>
<tr>
<td>Phosphate buffer ( pH-3)</td>
<td>40 %</td>
</tr>
<tr>
<td>Diluent</td>
<td>ACN: Methanol: phosphate buffer pH-3 (48:12:40 v/v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.2 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>25°C</td>
</tr>
<tr>
<td>Sample Size</td>
<td>20 µl</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>232 nm</td>
</tr>
<tr>
<td>Retention time</td>
<td>AML: 4.06 ± 0.5 min</td>
</tr>
<tr>
<td></td>
<td>OLM: 5.17 ± 0.5 min</td>
</tr>
<tr>
<td></td>
<td>HCZ: 2.61 ± 0.5 min</td>
</tr>
</tbody>
</table>

Table 3: Optical characteristics and linearity data of AML, OLM and HCZ

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>RP-HPLC Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AML</td>
</tr>
<tr>
<td>1</td>
<td>Working λ</td>
<td>232</td>
</tr>
<tr>
<td>2</td>
<td>Concentration (µg/ml)</td>
<td>5-25</td>
</tr>
<tr>
<td>3</td>
<td>Correlation Coefficient (r²)*</td>
<td>0.9997</td>
</tr>
<tr>
<td>4</td>
<td>Slope (m)*</td>
<td>64.92</td>
</tr>
<tr>
<td>5</td>
<td>Intercept (c)*</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

*Average of five determinations

Table 4: Response ratios of AML, OLM and HCZ

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (µg/ml)</th>
<th>AML</th>
<th>OLM</th>
<th>HCZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>328</td>
<td>65.6</td>
<td>348</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>656</td>
<td>65.6</td>
<td>689</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>985</td>
<td>65.6</td>
<td>1035</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>1305</td>
<td>65.4</td>
<td>1385</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>1632</td>
<td>65.4</td>
<td>1740</td>
</tr>
</tbody>
</table>

Table 5: Results of precision

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% MEAN ± SD*</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AML</td>
<td>OLM</td>
</tr>
<tr>
<td>Repeatability</td>
<td>98.88 ± 0.09</td>
<td>98.67 ± 0.04</td>
</tr>
<tr>
<td>Day to day precision</td>
<td>98.6 ± 0.06</td>
<td>98.57 ± 0.05</td>
</tr>
<tr>
<td>Analyst to Analyst</td>
<td>98.62 ± 0.04</td>
<td>98.34 ± 0.08</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>99.02 ± 0.04</td>
<td>99.38 ± 0.05</td>
</tr>
</tbody>
</table>

*Value of five replicate and five concentrations
Table 6: Results of recovery study

<table>
<thead>
<tr>
<th>% Level</th>
<th>AML</th>
<th>OLM</th>
<th>HCZ</th>
<th>% Mean ± SD*</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 %</td>
<td>98.45 ± 0.035</td>
<td>98.01 ± 0.035</td>
<td>98 ± 0.034</td>
<td>0.036</td>
<td>0.047</td>
</tr>
<tr>
<td>100 %</td>
<td>99.05 ± 0.045</td>
<td>98.71 ± 0.027</td>
<td>98.73 ± 0.033</td>
<td>0.046</td>
<td>0.028</td>
</tr>
<tr>
<td>120 %</td>
<td>98.67 ± 0.051</td>
<td>98.48 ± 0.033</td>
<td>98.9 ± 0.035</td>
<td>0.052</td>
<td>0.034</td>
</tr>
</tbody>
</table>

* Value of five replicate and five concentrations

Table 7: Results of robustness

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% Mean ± SD*</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robustness</td>
<td>98.34 ± 0.07</td>
<td>0.76</td>
</tr>
<tr>
<td>AML</td>
<td>98.65 ± 0.06</td>
<td>0.85</td>
</tr>
<tr>
<td>OLM</td>
<td>98.56 ± 0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>HCZ</td>
<td>0.76</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Value of five replicate and five concentrations

Table 8: Results and statistical parameters of tablet formulation

<table>
<thead>
<tr>
<th>Drug</th>
<th>Label Claim</th>
<th>Amount Found</th>
<th>MEAN*</th>
<th>S.D.*</th>
<th>%COV*</th>
<th>Std. Error*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>5</td>
<td>4.88</td>
<td>97.77</td>
<td>1.30</td>
<td>1.33</td>
<td>1.12</td>
</tr>
<tr>
<td>OLM</td>
<td>20</td>
<td>19.82</td>
<td>99.12</td>
<td>0.58</td>
<td>0.58</td>
<td>0.50</td>
</tr>
<tr>
<td>HCZ</td>
<td>12.5</td>
<td>12.38</td>
<td>99.06</td>
<td>0.67</td>
<td>0.68</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*Value of five replicate and five concentrations

Figure 1: (a) Structure of AML, (b) Structure of OLM and (c) Structure of HCZ

Figure 2a

Figure 2b

Figure 2c

Figure 2: (a) 2D Chromatogram (b) 3D view (c) Isoplot view of AML, OLM and HCZ.
CONCLUSION
In summary, we have developed and validated a rapid, specific, reproducible RP-HPLC method to quantify AML, OLM and HCZ simultaneously. So far no published methods are available for the simultaneous quantification of these three drugs in tablet dosage form. To the best of our knowledge, this is the first time that all three analytes were estimated simultaneously in any of the tablet dosage form. The cost-effectiveness, simplicity of the assay is that sample turnover rate of less than 7 minutes per sample; make it an attractive procedure in high-throughput analysis of AML, OLM and HCZ. From the results of all the validation parameters, we can conclude that the developed method can be useful for routine analysis and therapeutic drug monitoring with desired precision and accuracy.

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