ABSTRACT

A simple, accurate, precise, sensitive and stability indicating reverse phase high performance liquid chromatography method has been developed for simultaneous determination of amoxicillin trihydrate and cloxacillin sodium in bulk and in combined capsule dosage form. Chromatographic separation was performed on C\textsubscript{18} column, with mobile phase consist of water, acetonitrile, and methanol in the ratio of 70:20:10 (v/v/v), at flow rate 1.4 ml/min. Quantification of both drugs was achieved at UV detector at 238.8 nm. The retention time of amoxicillin trihydrate and cloxacillin sodium was found to be 8.240 and 14.036 minute respectively with run time 20 minutes. Amoxicillin trihydrate and cloxacillin sodium followed the linearity in concentration range 20-100 µg/ml for both with correlation coefficient ($r^2$) values 0.998 for amoxicillin trihydrate and 0.998 for cloxacillin sodium. The proposed method was validated according to ICH guidelines in terms of linearity, accuracy, precision, LOD and LOQ. Percentage assay was found to be 98.92 % and 99.61 % for amoxicillin trihydrate and cloxacillin sodium respectively. In precision % RSD was found to be < 2% for both. The percentage recovery was found in range 99.18%-99.42%. The LOD and LOQ values were within limit. The degradation studies carried under condition of acid, base, neutral, oxidative, photolysis, thermal degradation and no attempt was made to identify the degradation product.
KEYWORDS: Amoxicillin trihydrate, cloxacillin sodium, RP-HPLC, validation, stability indicating assay method.

INTRODUCTION

Amoxicillin trihydrate (AMO) is a broad spectrum semi-synthetic antibiotic. It is effective against a wide range of infections caused by wide range of Gram-negative bacteria and Gram-positive bacteria in human and animals. It acts by inhibiting synthesis of bacterial cell wall. Chemically AMO is (2S,5R,6R)-6-[(2R)-2-Amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicycloheptane-2-carboxylic acid.\(^1\)

Cloxacillin sodium (CLO) is semi synthetic antibiotic used against staphylococci that produce beta-lactamase. It is less active against pgN sensitive organisms. Chemically CLO (2S,5R,6R)-{[3-(2-chlorophenyl)-5-methyl-oxazole-4-carbonyl] amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.\(^2\) Structure of AMO and CLO are shown in figure 1.

According to literature survey AMO and CLOwas estimated by UV\(^{[3-11]}\), RP-HPLC\(^{[12-21]}\), HPTLC\(^{[22]}\), and stability indicating HPLC\(^{[23]}\) in combination, but no stability indicating RP-HPLC method has been reported for simultaneous estimation of AMO and CLO in combination by using water:acetonitrile:methanol (70:20:10) as mobile phase. Present work described the stability indicating HPLC method for simultaneous estimation of AMO and CLO in bulk and dosage form.

MATERIALS AND METHOD

Instrumentation and reagents

Chromatographic separation was performed on HPLC-system (model Shimadzu SCL- 10) C\(_{18}\) column (250 mm x 4.6 mm, 5 µm) UV Detector, equipped with a solvent delivery pump, sample injector and column thermostats. Lab solution (Version 1.25) software was applied for data collecting and processing. Water, acetonitrile, and methanolused were of HPLC grade. Pure drug sample of AMO and CLO was procured from Macleods Ltd., Daman.

Chromatographic condition

Mobile phase: Water: acetonitrile: methanol (70:20:10 v/v/v)

Column : C\(_{18}\)column

Detector wavelength : 238.8 nm
Injection volume : 20 µl
Flow rate : 1.4 ml/min
Run time : 20 minutes

Selection of detection of Wavelength
Accurately weighed 10 mg AMO and CLO were transferred into 100 ml volumetric flask separately, dissolved in mobile phase, sonicated and filtered through Whatman filter paper No. 41 and volume was made up to the mark with mobile phase. Pipette out 2 ml of this stock solution and diluted to 10 ml to get a concentration of 20 µg/ml of AMO and CLO each. The λ max was determined on Shimadzu UV-visible spectrophotometer (Shimadzu model UV-1800) in the range 200-400 nm. The overlain spectra showed iso-absorptive point at 238.8 nm was selected as wavelength. The overlain spectra of AMO and CLO are shown in figure 2.

Preparation of standard solution
Standard solution was prepared by transferring 50 mg of AMO and 50 mg of CLO in 50 ml of volumetric flask separately and dissolved in mobile phase. It was sonicated for 20 min to dissolve completely, and then volume was made up to the mark with mobile phase to get concentration 1000 µg/ml for both. From the above stock solution pipette out 1 ml and transferred to 10 ml volumetric flask and volume was made up with mobile phase and 20 µl was injected into HPLC system to develop the chromatogram. Typical chromatogram of AMO and CLO standard is shown in figure 3.

System Suitability test
System suitability was performed by injecting standard solution and determines the various parameters such as theoretical plates, tailing factor, resolution. Results are shown in table 1.

Method Validation
Method was validated as per ICH guidelines.

Linearity
Linearity was performed at five different concentration (20, 40, 60, 80, 100 µg/ml) for both AMO and CLO. Standard calibration curve was plotted between peak area against concentration of drug shown in figure 4 and results of linearity are shown in table 2.
Accuracy
The accuracy of the proposed method was performed by recovery studies at three different levels (80%, 100%, and 120%) of concentration by adding a known amount of standard drug to pre-analyzed sample. Each determination was repeated three times at each level and injected into HPLC system. Results of accuracy are shown in table 3.

Precision
Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation. The precision was determined at different parameter like repeatability, intermediate precision (intra-day, inter-day). Repeatability was determined by analyzing AMO (100 µg/ml) CLO (100 µg/ml) three times. Intraday precision was determined by analyzing same concentration of solution for three times within day and interday precision was determined daily for three days. Then % RSD was calculated and it was within limit (less than 2%). Results of precision are shown in table 4.

Limit of detection (LOD) and Limit of quantification (LOQ)
LOD is the lowest concentration of analyte in sample that can be detected but not necessarily quantified. LOQ is the lowest concentration of analyte in sample that can be quantitatively determined with precision and accuracy. Results of LOD and LOQ are given in table 5.

LOD and LOQ was calculated by using following formulae
LOD = 3.3 σ/slope,  LOQ = 10 σ/slope
(Where σ = the standard deviation of the response and S = slope of calibration curve).

Robustness
The robustness was performed by assaying test solutions after slight but deliberate change in analytical conditions. The study was performed by changing the flow rate, temperature and wavelength. Results of robustness study are shown in table 6.

Analysis of Marketed formulation
Twenty capsules weighed, average weight was determined, crushed to a fine powder and mixed thoroughly. Accurately weighed powder equivalent to 50 mg of AMO and 50 mg of CLO was transferred into 50 ml of volumetric flask and dissolved in mobile phase. This was sonicated for 20 minutes, and then volume was made up to mark with mobile phase. Further dilution was done with mobile phase to get final concentration of 100µg/ml of AMO and
100µg/ml of CLO. The standard and sample solution was injected into HPLC system to develop the chromatogram. Typical chromatogram of AMO and CLO is shown in figure 5. The content of AMO and CLO was calculated by using following formula. 

Amount of drug (mg) = At/As x Ds/Dt x Ws/Wt x A ……………..(i)  

% Estimation = At/As x Ws/Wt x Avg.wt (A)/Lable claim x 100 ……..(ii) 

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)  
A = Average weight of capsule  

Result are shown in table 7. 

**Forced Degradation Study**  
Stress degradation studies were performed to check the stability of the AMO and CLO on different conditions. The stress conditions for degradation study involved acid, base, neutral, oxidative, thermal, and photolytic degradation. 

**Acid degradation**  
Accurately weighed 20 mg of AMO and CLO was transferred to 100 ml volumetric flask, to it 20 ml mobile phase and 10 ml 0.1 N HCl was added. This flask was heated on water bath at 60°C for 4.30 hours. Solution was cooled and neutralized with 0.1 NaOH and volume was made up to mark with mobile phase, finally this solution was diluted with mobile phase to get concentration 20 µg/ml of AMO and 20 µg/ml of CLO. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition. 

**Base Degradation**  
Accurately weighed 20 mg of AMO and CLO was transferred to 100 ml volumetric flask, to it 20 ml mobile phase and 10 ml 0.1 N NaOH was added. This flask was heated on water bath at 60°C for 4.30 hours. Solution was cooled and neutralized with 0.1 N HCl and volume was made up to mark with mobile phase. Finally this solution was diluted with mobile phase to get
concentration 20 µg/ml of AMO and 20 µg/ml of CLO. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

**Oxidative degradation**

Accurately weighed 20 mg of AMO and CLO was transferred to 100 ml volumetric flask, to it 20 ml mobile phase and 10 ml 3% H₂O₂ was added. This flask was refluxed for 4 hours. Solution was cooled and volume was made up to mark with mobile phase, finally this solution was diluted with mobile phase to get concentration 20 µg/ml of AMO and 20 µg/ml of CLO. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

**Photolytic degradation**

Pure drugs were exposed to UV radiations for 12 hours. The sample after exposure to light were accurately weighed 20 mg of AMO and CLO was transferred to 100 ml volumetric flasks diluted with mobile phase to get concentration 20 µg/ml of AMO and 20 µg/ml of CLO. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

**Thermal degradation**

Thermal degradation was carried out by exposing pure drugs to dry heat at 80°C for 2 hours. The samples after exposure to heat were accurately weighed 20 mg of AMO and CLO, transferred to 100 ml volumetric flasks diluted with mobile phase to get AMO 20 µg/ml and CLO 20 µg/ml. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

**Neutral hydrolysis**

Accurately weighed 20 mg of AMO and CLO was transferred to 100 ml volumetric flask; to it 50 ml water was added. This flask was refluxed for 4 hours at 60°C, Solutions was cooled and volume was made up to mark with mobile phase. Finally this solution was diluted with mobile phase to get 20 µg/ml AMO and 20 µg/ml of CLO. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

Chromatogram obtained under various degradation conditions are shown in figure 6. Results of forced degradation studies are shown in table 8.
RESULT AND DISCUSSION
Several mobile phase compositions were tried to resolve the peak of AMO and CLO. The mobile phase water:acetonitrile:methanol (70:20:10) was found ideal to resolve the peak of AMO and CLO. The proposed method was found to be linear in concentration range of 20-100 µg/ml for both. Retention time of AMO and CLO were 8.240 and 14.036 min respectively. Percentage assay was found to be 98.92 and 99.61 for AMO and CLO. Systems suitability parameters were evaluated, which were within acceptance criteria. The accuracy of the method was confirmed by recovery studies at three different levels - 80%, 100%, 120%. In precision % RSD was found to be < 2% for AMO and CLO. The forced degradation showed AMO and CLO undergo degradation in acidic, basic, oxidative, photolytic, thermal, neutral condition and percentage degradation was found to be 7.48, 8.77, 7.1, 18.39, 4.23, 8.22, for AMO and 9.34, 11.05, 8.83, 17.43, 8.27, 12, for CLO.

(a) Amoxicillin trihydrate                      (b) Cloxacillin sodium

Fig. 1: Chemical structure of AMO and CLO.

Fig 2: Overlay spectra of AMO and CLO.
Fig 3: Chromatogram of standard solution.

Fig 4: Standard Calibration curve.

Fig 5: Chromatogram of AMO and CLO in marketed formulation.
a. Acid hydrolysis  
b. Base hydrolysis  

C. Oxidative degradation  
D. Photolytic degradation  

E. Thermal Degradation  
F. Neutral Degradation  

Fig 6: Forced degradation.
Table 1: Result of system suitability test

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Amoxicillin trihydrate</th>
<th>Cloxacillin sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>8.240</td>
<td>14.036</td>
</tr>
<tr>
<td>Peak area</td>
<td>303855</td>
<td>540700</td>
</tr>
<tr>
<td>Theoretical plates/meter</td>
<td>2216</td>
<td>3149</td>
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<tr>
<td>Tailing factor</td>
<td>1.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>1.4</td>
<td>1.5</td>
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<tr>
<td>Resolution</td>
<td></td>
<td>6.1</td>
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</table>

Table No 2: Observation for standard curve

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Amoxicillin conc. (µg/ml)</th>
<th>Peak area</th>
<th>Cloxacillin Conc.(µg/ml)</th>
<th>Peak area</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>61771</td>
<td>20</td>
<td>112084</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>126548</td>
<td>40</td>
<td>226168</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>179185</td>
<td>60</td>
<td>329952</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>248074</td>
<td>80</td>
<td>448336</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>303855</td>
<td>100</td>
<td>540700</td>
</tr>
</tbody>
</table>

Table 3: Result of Accuracy.

<table>
<thead>
<tr>
<th>Recovery level (%)</th>
<th>% Recovery*</th>
<th>Amount added(mg)</th>
<th>Amount Recovered (mg)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMO</td>
<td>CLO</td>
<td>AMO</td>
<td>CLO</td>
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<tr>
<td>80</td>
<td></td>
<td></td>
<td>39.67</td>
<td>39.8</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>40</td>
<td>49.79</td>
<td>49.71</td>
</tr>
<tr>
<td>120</td>
<td>60</td>
<td>50</td>
<td>59.83</td>
<td>59.65</td>
</tr>
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</table>

*Average of three determination.

Table 4: Result of precision

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMO</td>
</tr>
<tr>
<td>Precision (% RSD)*</td>
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</tr>
<tr>
<td>Repeatability</td>
<td>0.425</td>
</tr>
<tr>
<td>Intraday</td>
<td>0.433</td>
</tr>
<tr>
<td>Inter-day</td>
<td>0.425</td>
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</table>

*Average of three determination.

Table 5: Result of LOD and LOQ.

<table>
<thead>
<tr>
<th>Drug</th>
<th>LOD</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0.103</td>
<td>0.310</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>0.082</td>
<td>0.247</td>
</tr>
</tbody>
</table>

Table 6: Result of Ruggedness

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Parameter</th>
<th>Condition</th>
<th>Retention time (min)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flow rate</td>
<td>1.2 min/ml</td>
<td>9334</td>
<td>13.887</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 min/ml</td>
<td>8.240</td>
<td>14.036</td>
</tr>
<tr>
<td>2</td>
<td>Wavelength</td>
<td>236 nm</td>
<td>8.242</td>
<td>14.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td>238.8 nm</td>
<td>8.240</td>
<td>14.036</td>
</tr>
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</table>
Table 7: Analysis of Marketed Formulation

<table>
<thead>
<tr>
<th>Injection</th>
<th>Label claim (mg)</th>
<th>Assay (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin trihydrate</td>
<td>250 mg</td>
<td>98.92</td>
</tr>
<tr>
<td>Cloxacillin sodium</td>
<td>200 mg</td>
<td>99.61</td>
</tr>
</tbody>
</table>

*Average of three determination.

Table 8: Result of Forced Degradation study.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Condition</th>
<th>% Degradation</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AMOX</td>
<td>CLOX</td>
</tr>
<tr>
<td>01.</td>
<td>Acid hydrolysis</td>
<td>7.48</td>
<td>9.34</td>
</tr>
<tr>
<td>02.</td>
<td>Base hydrolysis</td>
<td>8.77</td>
<td>11.05</td>
</tr>
<tr>
<td>03.</td>
<td>Oxidative degradation</td>
<td>7.71</td>
<td>8.33</td>
</tr>
<tr>
<td>04.</td>
<td>Photo degradation</td>
<td>18.39</td>
<td>17.43</td>
</tr>
<tr>
<td>05.</td>
<td>Thermal degradation</td>
<td>4.23</td>
<td>8.27</td>
</tr>
<tr>
<td>06.</td>
<td>Neutral degradation</td>
<td>8.22</td>
<td>12</td>
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</table>

CONCLUSION

The developed HPLC method is found to be simple, specific, accurate and stability indicating, hence it can be used for routine quality control analysis as well as stability studies for the estimation of amoxicillin trihydrate and cloxacillin sodium in bulk and in combined capsule dosage form. The degradation of AMO and CLO was determined by subjecting them in various stress condition and no attempt was made to identify the degradation product.

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