DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHOD FOR THE DETERMINATION OF AFLOQUALONE IN HUMAN PLASMA AND FORMULATION

M.V. Basaveswara Rao*, V. Prasanthi, Sushanta Maiti and G. Raja

1Department of Chemistry, Krishna University, Machilipatnam, Andhra Pradesh, India.

*Corresponding Author: vbrmandava@yahoo.com, professormandava@gmail.com

INTRODUCTION

Afloqualone is an analogue of methaqualone and has a sedative and muscle relaxant effects. 6-amino-2-(fluoromethyl)-3-(2-methylphenyl)-4(3H)-quinozolinone is official in Indian Pharmacopoeia. Its molecular formula is C16H14FN3O and molecular weight is 283.30. The literature survey indicates there are several methods reported for the determination of Afloqualone in human plasma and formulations by RP-HPLC. Ochiai T and Ishida R. reported the Pharmacological studies on afloqualone, and its spinal reflex potential. Ishikawa T, Kamide R, Niimura M. studied the Photoleukomelanodermatitis (Kobori) induced by afloqualone. Miura Y, Kono T, Chishima S, Takeyama S, Arami S, and Sakaguchi K reported the Plasma drug concentrations and clinical observations after single or repeated oral administration of afloqualone in healthy volunteers. Otsuka M, Furuuchi S, Usuki S, Nitta S, and Harigaya S described the Metabolism of afloqualone, a new centrally acting muscle relaxant, in monkeys and dogs. Otsuka M, Kurozumi T, Furuuchi S, Usuki S, Koteru K, and Harigaya S reported the metabolism of afloqualone, in the rat. Potential developments and new approaches in controlled release drug delivery systems are reported.

ABSTRACT

A simple, rapid and precise reverse phase high performance liquid chromatography method has been developed and validated for the determination of Afloqualone in tablet and serum. Chromatography was performed by Shimadzu Model LC-20 AT VP with Kromasil C-18 column 250 x 4.6 mm ID with 5 µ particle sizes and the column maintained at ambient temperature. The injection volume was 20µl and the total run time was 6 min. The detection was carried out at 285 nm. The mobile phase consisted of Acetonitrile, 1% Acetic acid and Water (40:40:20 v/v). Prepared mobile phase was filtered through 0.45µ membrane filter and sonicated. Sample solution was prepared by dissolving the drug in mobile phase and sonicated for 30 minutes. The mobile phase was delivered isocratically at a flow rate of 1ml/ min.

Keywords: Afloqualone, RP-HPLC, Kromasil C-18 column.
an attempt was made to develop and validate a simple, precise, accurate and economical RP-HPLC method as per ICH guidelines for the estimation of Afloqualone in pure pharmaceutical dosage forms and to apply the developed method to determine the forced degradation compounds.

**Materials and Methods**

**Preparation of Mobile Phase Solution**
The mobile phase was prepared by mixing Acetonitrile and 1% Acetic acid and Water (40:40:20 v/v) by ultra bath sonicator for 30 min.

**Preparation of Standard**
Stock solution of Afloqualone was prepared by dissolving accurately weighed 10mg of drugs in 10ml Methanol (final concentration, 1mg/ ml). The prepared stock solutions were stored away from light. From the stock, standard solutions was freshly prepared during the day of analysis.

**Preparation of Working Standard solution (A.P.I)**
From the stock solution 20 µg/ ml solution was prepared.

**Linearity and Calibration**
Linearity was assessed by performing single measurement at several analyte concentration varying quantities of stock standard solution diluted with the mobile phase to give a concentration of 1, 2, 3, 4, 5 µg/ ml injection was made at intervals of 6 min. The linearity was tested for the concentration ranging from 1µg/ ml to 5 µg/ ml. The peak area ratio of the drug was plotted against concentration. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

**RESULT AND DISCUSSION**
The Reverse Phase High Performance Liquid Chromatography method was developed by a stability indicating assay method. Pure drugs chromatogram was run in different mobile phases containing methanol, acetonitrile, THF, and different buffers like potassium dihydrogen phosphate, sodium dihydrogen phosphate, Ortho phosphoric acid in different volumes ratios. Different columns like C₈, C₁₈, phenyl, cyano with different dimensions were used. Then retention time and tailing factor were calculated. Finally Acetonitrile and 1% Acetic acid and Water in the volume of ratio 40:40:20 v/v (pH: 4.82) and Kromosil C₁₈ analytical column was selected which gave a sharp and symmetrical peak with 1.84 tailing. Calibration graph was found to be linear at range 1µg/ ml to 5 µg/ ml. five different concentrations of Afloqualone in range given above were prepared and 20 µl of each concentration injected in HPLC. The slope (m) and intercept (c) obtained were found to be 6073.59 and 0.072465428. The correlation of coefficient (r²) obtained was found to be 0.9996. It was observed that the concentration range showed a good relationship. The limit of detection for Afloqualone was found to be 10µg/ ml and the limit of quantification was found to be 30µg/ ml. It proves the sensitivity of the method. The Percentage assay of Afloqualone in formulation was found to be 100.59%. The relative standard deviation value obtained was below 1 which indicates the precision of the method. The validation of the proposed method was further verified by recovery studies. The percentage recovery was found to be 102.92% which shows a good index of accuracy of the developed method. The amount of drug present in the human serum sample was calculated from the linearity graph was found to be 4.576mg/ 5ml.

**Precision**
Reproducibility was performed by injecting three replicates concentrations of standard and sample solutions which were prepared and analyzed by same analyst on same day. Inter-day variations in the peak area of drug solutions and the amount of drug were calculated in terms of Percentage Relative Standard Deviation. The sample concentration is 20µg/ ml.

**Accuracy**
Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 80%, 100% and 120% to the pre analyzed sample formulation.

**Intermediate Precision or Ruggedness**
Inter-day variations were performed by using six replicate injections of standard and sample solutions of concentrations which were prepared and analyzed by different analyst on three different days over a period of one week. Ruggedness also expressed in terms of percentage relative standard deviation.
Robustness
Robustness was carried out by varying two parameters from the optimized chromatographic conditions.

Specificity
The method was determined as specific by comparing test results obtained from analyses of sample solution containing excise ingredients with that of test results those obtained from standard drug.

System Suitability Parameter
System suitability tests were carried out on freshly prepared standard stock solutions of Afloqualone and it was calculated by determining the standard deviation of Afloqualone standards by injecting standards in five replicates at 6 minutes interval and the values were recorded.

Assay
The estimation of drug in pharmaceutical dosage forms AROFUTO 100mg strength was evaluated for the amount of Afloqualone present in the formulation. Each sample was analyzed in triplicate after extracting the drug. The amount of drug present in formulation was calculated by comparing the mean peak area from standard.

Preparation of Formulation Sample Solution
5mg of formulation powder was taken from AROFUTO (100 mg formulation) and dissolved in 5ml of mobile phase and injected into HPLC and chromatogram was recorded.

Preparation of Serum Sample Solution
From a local hospital blood was collected and serum was separated. 5ml of this serum was taken in a test tube and added 100µl of diltiazem hydrochloride (1µg/ ml) and 0.1ml of 1M NaOH and 5ml of dichloromethane and mixed about 20min in vortex mixer and centrifuged at 3000 rpm for 10min. From this centrifuged solution 4ml of organic layer was separated and evaporated to dryness to get residue. To this residue 100µl of 1M acetic acid and 3ml of n-Hexane and mixed for 5 min by vortex mixer and evaporated the organic layer and finally the remaining sample was injected into HPLC and chromatogram was recorded. The amount of drug present in the blood sample was calculated from linearity graph.

Conclusion
The RP-high performance liquid chromatographic method for the analysis of Afloqualone from their formulations was found to be accurate and precise. Thus, the proposed HPLC method can be successfully applied for the routine quality control analysis of Afloqualone formulations.

Acknowledgements
The authors are thankful to the University Grants Commission for the financial assistance under the Major Research Project.

Table 1: Optical Characterization of Afloqualone

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>AFLOQUALONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range(µg/ ml)</td>
<td>1 - 5</td>
</tr>
<tr>
<td>Correlation coefficient (r )</td>
<td>0.9996</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>60073.59</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>0.072465428</td>
</tr>
<tr>
<td>Limit of detection (LOD; µg/ ml)</td>
<td>10</td>
</tr>
<tr>
<td>Limit of Quantification (LOQ; µg/ ml)</td>
<td>30</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.21</td>
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<tr>
<td>Retention time (min)</td>
<td>1.671</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>2110</td>
</tr>
<tr>
<td>(%) R.S.D</td>
<td>0.044</td>
</tr>
<tr>
<td>(%) Accuracy</td>
<td>102.92</td>
</tr>
<tr>
<td>FORMULATION ASSAY( % )</td>
<td>100.59</td>
</tr>
<tr>
<td>SERUM (mg/ 5ml)</td>
<td>4.576</td>
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</tbody>
</table>
Table 2: Recovery Data of Afloqualone

<table>
<thead>
<tr>
<th>PHARMACEUTICAL FORMULATION (Brand Name)</th>
<th>LABELED AMOUNT (mg)</th>
<th>PERCENTAGE ASSAY</th>
<th>PERCENTAGE RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>AROFUTO</td>
<td>100 mg</td>
<td>100.59</td>
<td>102.92</td>
</tr>
</tbody>
</table>

*Average value of three different levels in triplicate

HPLC Report

ID | Name     | Retain T | Height | Area  | Conc  | Tail Factor | Theo Plate |
---|----------|----------|--------|-------|-------|-------------|------------|
1  | Afloqualone | 1.520    | 10145  | 52824.4 | 100.000 | 0.85         | 4299       |
Sum: | Afloqualone | 10145  | 52824.4 | 100.000 | 0.85 | 4299 |

Fig. 1: Standard Chromatogram

Fig. 2: Sample Chromatogram
Fig. 3: Serum Chromatogram

REFERENCES


