DEVELOPMENT AND VALIDATION OF A METHOD FOR QUANTITATIVE DETERMINATION OF AMIODARONE HYDROCHLORIDE IN BLOOD SERUM BY HPLC-MS-MS

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DEVELOPMENT AND VALIDATION OF A QUANTITATIVE METHOD FOR THE EVALUATION OF AMIODARONE HYDROCHLORIDE BY HPLC-MS-MS IN BLOOD SERUM (Abstract) 
Aim: The paper presents the development and validation stages of a new method using high performance liquid chromatography coupled with mass spectrometry for the determination of amiodarone hydrochloride in blood serum. Material and methods: The method used a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer coupled with a HPLC Transcend TLX1 system and octadecylsilyl silica gel C₁₈ Cyclone MAX polymeric chromatographic columns (50 mm x 2.1 mm; 1.9 µm). Mass spectrometry detection was based on transition of ions from 645.9 to 200.23 m/z, while the other parameters were: 2400 V ionization potential, 10 psi pressure of gas ionization, 60 psi auxiliary gas pressure, 2.8 mTorr energy collision, 200°C ionization temperature and 330°C capillary temperature. A thorough validation study was conducted that included identifying the characteristic peaks of amiodarone hydrochloride, evaluating system compatibility, studying linearity and accuracy, establishing chromatographic retention times, limits of detection and quantification. Results: The retention time for amiodarone hydrochloride was 2.42 minutes, the method was linear over the concentration range 0.05-1.25 mg/mL, the limit of detection was 5.38 mg/mL, the limit of quantification was 17.68 mg/mL, and the regression coefficient was $r² = 0.9986$. Conclusions: The HPLC-MS-MS method developed and validated for the quantitative determination of amiodarone hydrochloride in serum is fast, specific and simple, and it can be used with good results during the in vivo studies required for the development of new therapeutic systems of amiodarone. Keywords: AMIODARONE, HPLC-MS-MS, BLOOD SERUM.

Amiodarone (AMD) is one of the most frequently prescribed therapeutic agents for the treatment of cardiac tachycardia worldwide due to its unanimously recognized efficiency. The mechanism of action includes the prolongation of action potential and effective refractory period of the myocardial fibers especially in the atria and His-Purkinje system, but also in the ventricular myocardium, and a decrease in sinus node automatism. AMD determines atrioventricular conduction delay, de-
creased heart rate and significant lengthening of the Q-T interval on standard electrocardiogram (1, 2, 3).

The physicochemical properties of AMD were at the basis of this analytical study in order to develop new quantitative methods consistent with drug control regulations for bulk substance and solid oral dosage forms with modified and immediate release (4, 5).

For the quantitative analysis of AMD conditioned into modified release solid oral pharmaceutical formulations F1 and F10 or as amiodarone/hydroxyl-propyl-β-cyclodextrine inclusion complex (HP-β-CD/AMD), we have previously developed and validated a new HPLC method for the assay of AMD for in vitro studies. An exhaustive study on the quantitative determination methods of AMD from raw materials, formulations and biological fluids was at the basis of the method presented in this article. Most of the reported methods either do not include stress degradation studies, are validated incompletely, or are time consuming, cumbersome and expensive. For that reason a novel method using HPLC coupled with MS was developed and validated, method that is fast, specific and easy to use for the quantitative determination of AMD in blood serum (6-8).

MATERIAL AND METHODS

The reagents used were: methanol HPLC grade (Merck, Germany), formic acid, ammonium formate (Merck, Germany), acetonitrile, isopropanol, acetone HPLC grade (Merck, Germany), HP-β-CD - 99.70% purity (Roquette France), amiodarone hydrochloride (AMD-HCl) - 99.85% purity (Zhejlang Pharmaceutical Co. Ltd., China), HP-β-CD/AMD complex - 99.80% purity ("Petru Poni” Institute of Macromolecular Chemistry Iasi, Romania), Millipore-filtered water - 0.01 μS/cm conductivity.

The solution used were: sample solution (1 mL serum sample plus 2 mL of acetonitrile were sonicated for 5 minutes and then centrifuged for 10 minutes at 4000 rpm and decanted off), 0.01 mg/mL AMD standard solution (100 mg AMD-HCl were dissolved in water and diluted to 100 mL, and then 0.1 mL were diluted with mobile phase up to 10 mL).

The chromatographic parameters used were: chromatographic column 1 - C\textsubscript{18} octadecylsilyl silica gel (0.05 m x 2.1 mm, 1.9 μm, 25°C), chromatographic column 2 - polymeric Cyclone MAX (0.05 m x 2.1 mm, 1.5 μm, 50°C), mobile phase A (for C1) 0.1 mM ammonium formiate and 0.001% formic acid, mobile phase B – water, mobile phase C - methanol, mobile phase D - acetonitrile: isopropanol: acetone (40:40:20) (v/v/v) (last 3 for C2). The volume of sample injected was 5 μL.

The quantitative determination of AMD was based on the external standard method. The method was based on separation of the analyte from the biological matrix through chromatographic techniques with mass spectrometry detection.

The chromatographic method of analysis included two phases. First, the distribution of the analyte was carried out through column 1 which provided a turbulent flow, and then the analyte was transferred to column 2 for separation and identification by MS. The column was prepped with the mobile phase at a flow rate of 0.5 mL/min for 30 minutes.

**Validation procedure**

The chromatographic peaks were identified by injecting a solution containing the active substance AMD in the biological matrix – blood serum.

Chromatographic system compatibility study was done by injecting 10 times con-
secutively 200 ng/mL of AMD solution. Injection repeatability, reflected in the invariability of both peak area and retention times, defined the accuracy of the system. Also peak asymmetry and number of theoretical plates in the column were two other factors that characterized system compatibility.

The linearity study of the signal corresponding to AMD·HCl was done by plotting the calibration curve over the linearity range 0.05-1.25 µg/mL. Three independent series of these solutions for each concentration level have been used (9,10).

In order to study the accuracy of the analysis method, spiked samples were prepared containing various amounts of AMD·HCl dissolved in a mixture of 500 mL plasma and 500 mL water, as follows: SP1 - 1250 ng AMD, SP2 - 1000 ng AMD, SP3 - 500 ng AMD, SP4 - 200 ng AMD, SP5 - 75 ng AMD, SP6 - 50 ng AMD. The recovered concentration (C\text{recovered}), percentage recovery (R\%), and percent deviation (Xd\%) were calculated, while the obliquity of the experiment was evaluated.

For the calculation of the limit of detection (LOD) and limit of quantification (LOQ), the standard deviation and the slope of the regression curve were used according to the following equations: LOD = 3.3 × SD/slope and LOQ = 10×SD/slope (11-12).

**RESULTS AND DISCUSSION**

The results obtained for the identification of the corresponding peaks of AMD·HCl (fig. 1-4) are reported in table I.

The experimental results obtained in the linearity study are presented in table II.

Estimation of peak areas had been done using linear regression equation: Area = 4.142 × Concentration.

The fitting of peak areas based on the variation of residual values according to the concentration of AMD·HCl is shown in table III.

The experimental results of the accuracy study are presented in table IV.

The experimental results of the linearity study are presented in table V.

**TABLE I**

Identification of the characteristic peaks of AMD·HCl

<table>
<thead>
<tr>
<th>Solution injected</th>
<th>AMD·HCl concentration (µg/mL)</th>
<th>Retention time (minutes)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI1</td>
<td>500</td>
<td>2.42</td>
<td>1984</td>
</tr>
<tr>
<td>Blank</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Fig. 1. Peak of 1250 ppb reference AMD](image1)

![Fig. 2. Peak of AMD inclusion complex](image2)
TABLE II
Linear variation of peak area with sample concentration

<table>
<thead>
<tr>
<th>( C_{\text{AMD-HCl}} ) (ppb)</th>
<th>50</th>
<th>75</th>
<th>200</th>
<th>500</th>
<th>1000</th>
<th>1250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean area</td>
<td>120.00</td>
<td>240.50</td>
<td>835.51</td>
<td>2088.77</td>
<td>4472.63</td>
<td>5535.44</td>
</tr>
</tbody>
</table>

TABLE III
Fitting residual values

<table>
<thead>
<tr>
<th>No.</th>
<th>Predicted values</th>
<th>Residual values</th>
<th>Standard residual values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83.87788</td>
<td>-23.8779</td>
<td>-0.54378</td>
</tr>
<tr>
<td>2</td>
<td>192.2334</td>
<td>-7.23343</td>
<td>-0.16473</td>
</tr>
<tr>
<td>3</td>
<td>734.0112</td>
<td>59.72215</td>
<td>1.360064</td>
</tr>
<tr>
<td>4</td>
<td>2034.278</td>
<td>-49.9444</td>
<td>-1.13739</td>
</tr>
<tr>
<td>5</td>
<td>4201.389</td>
<td>47.61122</td>
<td>1.084259</td>
</tr>
<tr>
<td>6</td>
<td>5284.944</td>
<td>-26.2776</td>
<td>-0.59843</td>
</tr>
</tbody>
</table>

TABLE IV
Study on method accuracy

<table>
<thead>
<tr>
<th>Sample</th>
<th>Theoretical concentration (ng/mL)</th>
<th>( C_{\text{recovered}} ) (ng/mL)</th>
<th>R%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>50</td>
<td>48</td>
<td>96</td>
</tr>
<tr>
<td>SP2</td>
<td>75</td>
<td>72</td>
<td>96</td>
</tr>
<tr>
<td>SP3</td>
<td>200</td>
<td>196</td>
<td>98</td>
</tr>
<tr>
<td>SP4</td>
<td>500</td>
<td>490</td>
<td>98</td>
</tr>
<tr>
<td>SP5</td>
<td>1000</td>
<td>970</td>
<td>97</td>
</tr>
<tr>
<td>SP6</td>
<td>1250</td>
<td>1191</td>
<td>95</td>
</tr>
</tbody>
</table>
Development and validation of a method for quantitative determination of amiodarone hydrochloride in blood serum by HPLC-MS-MS

TABLE V
Linearity of the method

<table>
<thead>
<tr>
<th>C_{AMD·HCl}</th>
<th>50</th>
<th>75</th>
<th>200</th>
<th>500</th>
<th>1000</th>
<th>1250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area(S1)</td>
<td>62.00</td>
<td>185.00</td>
<td>793.73</td>
<td>1984.33</td>
<td>4249.00</td>
<td>5258.67</td>
</tr>
<tr>
<td>Area(S2)</td>
<td>68.54</td>
<td>186.00</td>
<td>794.73</td>
<td>1984.22</td>
<td>4334.00</td>
<td>5345.00</td>
</tr>
<tr>
<td>Area(S3)</td>
<td>53.75</td>
<td>184.00</td>
<td>792.55</td>
<td>1953.40</td>
<td>4149.00</td>
<td>5144.00</td>
</tr>
<tr>
<td>Average</td>
<td>60.30</td>
<td>185.00</td>
<td>793.50</td>
<td>1973.98</td>
<td>4244.00</td>
<td>5249.22</td>
</tr>
</tbody>
</table>

The limits of detection and quantification were calculated based on the response to the lowest concentration for which the relative standard deviation has been determined, and it was then compared to the slope. The values of the limit of detection and limit of quantification were found to be 5.83μg/mL and 17.68 μg/mL, respectively.

The method was validated in accordance with international regulations (13,14). The representative chromatograms of AMD free and spiked plasma samples showed no secondary peaks of interfering substances such as endogenous plasma components, with similar retention time. The retention time of the analyte was 2.42 minutes.

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All accuracy and precision values were within the recommended range. The degree of recovery ranged from 95% to 98% (15,16).

Injection repeatability, peak area and retention times defined system accuracy. Also peak symmetry and number of theoretical plates of the column were two other factors that characterized the compatibility of the system. Injection repeatability was proven by RSD ≤ 2.

Under the experimental conditions, it was observed that the confidence interval for the average area (793.67) was $X = -362.87 \div 1950.20$ and RSD%_{AMD} (area) was 1.59. Stretch factor was $T \leq 1.8$, and $T_{AMD} = 1.1$.

The number of theoretical plates for AMD-HCl was $N_{AMD} = 3757$. Calibration curve showed a linear variation of the peak area with sample concentration. During accuracy study, the experimental data highlighted an 80.48% average recovery (17,18).

CONCLUSIONS
We validated the new method in a 0.05-1.25 μg/mL concentration range, covering the therapeutic plasma concentration of amiodarone hydrochloride.

The developed method developed is simple, fast, and accurate and it did not involve great cost. Compared to other published HPLC methods for the assay of amiodarone hydrochloride in animal plasma, the new method performed better in terms of speed and cost, important characteristics for the methods used routinely.

We have successfully used the newly validated method in a pharmacokinetic study, but it may also have widespread applications in monitoring AMD therapeutic concentration in blood serum during studies investigating drug interactions and in toxicological research.

REFERENCES


