**Objective:** The purpose of this study was to develop a LC-MS method to determine amiodarone (AMI) and its major metabolite desethylamiodarone (DEA) from rat plasma released from the adipose tissue of AMI treated rats subjected to a weight gain/weight loss cycle.

**Methods:** Separation of the compounds was performed on a Kinetex 2.6 μm C18 100 x 4.6 mm column under isocratic conditions using a mixture of acetonitrile: 0.1% formic acid 65:35 at a flow rate of 0.5 ml/min. Detection of the analyte was performed by electrospray positive ionization, the monitored ions being 135 m/z from 646 for AMI and 135 m/z of 618 for DEA. Analytes were extracted after plasma protein precipitation with methanol.

**Results:** The developed method presented specificity and linearity on the concentration range of 25-2500 ng/ml plasma for AMI and 2.5-1250 ng/ml plasma for DEA and the precision and accuracy of the method at all of quality control concentration levels including LLOQ were according to official guidelines for validating analytical methods.

**Conclusions:** A sensitive and accurate LC-MS method has been developed with a much lower LLOQ than literature data to detect the plasma concentration differences of the studied analytes that result from forced lipolysis and mobilization from the adipose tissue.

**Keywords:** amiodarone, desethylamiodarone, rat, lipolysis

**Introduction**

Amiodarone (AMI) is a compound with a particular pharmacodynamic and pharmacotoxicological profile among cardiovascular medication. In addition to antiarrhythmic action as a K⁺ channel blocker, it also presents non-competitive antagonistic action on alpha and beta receptors. In clinical practice, AMI is used to control ventricular and supraventricular tachycardia but also for the prevention of recurrences. AMI increases life expectancy in patients post myocardial infarction or suffering from congestive heart failure [1]. From the pharmacokinetic point of view, it has a variable oral absorption, a bioavailability of 22 - 86% and a high volume of distribution. The half-life is 5-20 hours after a single dose, but is up to 58 days after a long-term treatment [2].

Desethylamiodarone (DEA) is the main metabolite of AMI and presents similar therapeutic and toxicological properties to AMI. Even if plasma concentrations of the metabolite are comparable to those of the active substance, in some tissues, there are differences in concentrations. Until the steady state is established, the active substance and the metabolite accumulate in the tissues. Long-term administration of AMI produces excessive accumulation through a mechanism that can not be explained by classical (single compartment model) pharmacokinetics and involves increasing tissue and plasma concentration by maintaining the initial dose. Nowadays, the use of AMI is often associated with a variety of adverse effects, causing patient noncompliance because of the severe toxicity. Multiple organs are affected, including the lungs [3], liver [4], thyroid gland [5], neurological system, skin and gastrointestinal system. Some of these side effects are reversible, but severe cases have also been reported. The fundamental mechanism of AMI toxicity is not fully known, systemic toxic effects are supposed to be related to the accumulation of phospholipids in the target tissues [6].

Despite the multiple adverse effects, because of the low treatment cost, AMI is the antiarrhythmic of choice in ventricular and supraventricular tachycardia, but due to the tendency of cumulation in different organs, in case of intense lipolysis (diet with the aim of reducing body weight or forced in the case of a stroke or post-acute myocardial infarction) massive release from deposits can lead to exacerbation of adverse effects. Under these circumstances, the aim of the present study is to develop a sensitive and precise LC-MS method to determine AMI and DEA from rat plasma, that can quantify the excess of AMI and DEA released from the deposits in the adipose tissue, following lipolysis in obese animals treated with AMI.

**Materials and Methods**

**Chemicals, reagents, solvents**

AMI and DEA standards were purchased from Sigma Aldrich and were > 98% pure. Methanol, acetonitrile and formic acid of analytical grade were purchased from Merck KGaA (Darmstadt, Germany). Distilled water was obtained from a Millipore Direct Q system.
Preparation of standard solutions
The AMI and DEA 1 mg/ml stock solutions were prepared by weighing 10.56 mg AMI hydrochloride (corresponding to 10 mg AMI) on a Metler Toledo AB 54-S balance which was dissolved in 10 ml of methanol, respectively, 5.29 mg of DEA hydrochloride (corresponding to 5 mg of DEA) dissolved in 5 ml of methanol. Standard work solutions, at 8 concentration levels, were prepared by diluting stock solutions with methanol over the concentration range of 25-2500 ng/ml for AMI and 2.5-1250 ng/ml for DEA. Also, 3 quality control samples (QC) of 75, 1250 and 2000 ng/ml for AMI and 37.5, 625 and 1000 ng/ml for DEA were prepared.

Plasma sample processing
Rat plasma samples were collected by cardiac puncture, in EDTA anticoagulant vials. All procedures on experimental animals were performed in agreement with the Ethics Committee for Scientific Research of the University of Medicine and Pharmacy of Tîrgu Mureș. The plasma calibration curve was constructed on the concentration range of 5-500 ng/ml plasma for AMI and 2.5-250 ng/ml plasma for DEA.

For the calibration curve and plasma QC samples, 120 μl rat plasma was spiked with 40 μl AMI solution and 40 μl DEA solution. Plasma proteins were precipitated with 600 μl methanol and the samples were vortex-mixed for 10 seconds. The mixture was centrifuged for 10 minutes at 10000 rpm. 500 μl supernatant was transferred to a chromatography vial and 5 μl were injected into the chromatographic system.

Chromatographic conditions
Separation of the compounds was performed on an Agilent 1100 Series System (Agilent Technologies, USA) consisting of a quaternary pump, solvent degasser, autosampler with controlled temperature, column thermostat and an Agilent 6410 QQQ mass spectrometer detector with ESI source. Separation was performed on a Kinetex 2.6 μm C18 100 x 4.6 mm column. The mobile phase used for the separation consisted from acetonitrile and 0.1% formic acid in 65:35 isocratic mixture, with a flow rate of 0.5 ml/min.

MS conditions:
- ionization type and mode: ESI+;
- nebulizer nitrogen: 40 psi;
- drying gas: nitrogen, flow rate 8 L/min;
- source temperature 350°C;
- capillary potential: 4000 V;
- monitoring transitions m/z 646 → 135 for AMI and 618 → 135 for DEA.

Results and discussions
AMI determination was performed by molecular ion fragmentation [M+H]⁺ m/z 646 and DEA starting from [M+H]⁺ m/z 618 to the m/z 135 fragment for both compounds (Figure 1).

Chromatograms of rat plasma samples spiked with AMI and DEA at the lower limit of quantification (LLOQ) are presented in Figure 2 and 3. Compounds of interest are separated at a retention time of 3.94 min AMI and 3.16 min DEA.

HPLC method performances

Specificity studies
The specificity of the method was verified using 6 blank rat plasma samples.

Linearity studies
3 calibration curves were prepared, with 8 concentration levels, in rat plasma and the medium calibration curves of

Fig. 1. Full-scan mass spectrum of AMI and DEA in the mobile phase
Fig. 2. Chromatogram of a blanc plasma sample (blue line) and an AMI-spiked sample at LLOQ (5 ng/ml plasma)

Fig. 3. Chromatogram of a blank plasma sample (black line) and spiked with DEA at LLOQ (2.5 ng/ml plasma)

Fig. 4. Chromatogram of a rat plasma sample spiked with AMI and DEA at LLOQ (5 ng/ml plasma AMI and 2.5 ng/ml plasma DEA)
y = a (± SD) x + b (± SD) type, where y-concentration and x-peak area, were y = 1462.40 (±72.95) x + 4679.66 (± 2400.32) for AMI with a determination coefficient R² > 0.99 and y = 1464.20 (±129.51) x + 15907.33 (±3803.84) for DEA with a determination coefficient R² > 0.99.

Residuals distribution was random and within ±15% limits. The results demonstrated a good fitting calibration model over the selected concentration range and in accordance to bioanalysis quality criteria.

**Precision and accuracy**

The precision within- and between-run (CV%) and accuracy (Bias%) were determined during the same series and on different days, on 5 individual LLOQ, QCA, QCB and QCC samples, at concentrations of 5, 15, 250, 400 ng/ml plasma AMI and 2.5, 7.5, 125, 200 ng/ml plasma DEA, according to validation guidelines.

The method presents a good accuracy (101.45% between-run and 97.68% within-run for DEA and 97.68% between-run and 98.57% within-run for DEA and 97.68% between-run and 104.76% between-run for AMI) and precision for both compounds (9.46% within-run and 11.12% between-run for AMI and 13.56% within-run and 14.23% between-run for DEA) (Table I and II).

LLOQ was set at 5 ng/ml plasma AMI and 2.5 ng/ml plasma DEA, therefore the LC-MS method developed for AMI and DEA determination in rat plasma presents a much lower LLOQ compared to methods already published in scientific literature [7, 8, 9].

Starting from these premises, the developed method will be used in the study of AMI and DEA plasma levels following chronic treatment in rats undergoing a controlled weight gain/weight loss cycle and compared to a control group (with no significant fluctuations in weight during the experiment). The animal model was chosen according to the similar pharmacokinetic properties of AMI in the two species. Although the microsomal enzyme system differs between human and rat, DEA was confirmed as the active metabolite in both cases [10, 11].

As in the future we aim to determine the plasma concentrations of the two compounds at different time intervals, until complete elimination, an analytical method with increased sensitivity is required.

In addition, in order to demonstrate the applicability of analytical methods, animals received doses of AMI between 25 and 200 mg/kg bw (intraperitoneal or intravenous) in bolus; in efficacy and safety studies similar oral doses were administrated. However, in the case of oral administration, the plasma concentrations obtained are much lower compared to those obtained after parenteral administration.

Although toxicity mechanisms may vary depending on the organ, numerous studies have shown that the occurrence of side effects is associated with the duration of treatment and excessive accumulation of AMI in the body.

Due to increased lipophilia, AMI is predominantly distributed in adipose tissue with a particular pharmacokinetics in obese patients with frequent cardiac abnormalities, obesity being associated with a pro-inflammatory condition and increased oxidative stress. Administration of AMI to these patients should be made with caution considering the individual variability of pharmacokinetics and the impossibility to monitor plasma concentrations during chronic treatment [12].

In weight-loss diets (obese patients with increased cardiovascular risk at which gradual weight loss is recommended), or in case of forced weight-loss (parenteral nutrition in patients unable to receive a balanced nutritional and caloric intake after myocardial infarction or stroke) by

**Table I. Within- and between-run precision, accuracy and recovery for AMI (n = 5)**

<table>
<thead>
<tr>
<th>C nominal ng/ml</th>
<th>Mean C ng/ml (±SD)</th>
<th>Recovery % (±SD)</th>
<th>CV% (Bias%)</th>
<th>Mean C ng/ml (±SD)</th>
<th>Recovery % (±SD)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (±0.41)</td>
<td>4.88</td>
<td>97.68</td>
<td>9.46</td>
<td>5.23</td>
<td>104.76</td>
<td>11.12</td>
</tr>
<tr>
<td>15 (±0.86)</td>
<td>15.08</td>
<td>100.51</td>
<td>5.74</td>
<td>14.36</td>
<td>95.76</td>
<td>12.08</td>
</tr>
<tr>
<td>250 (±3.73)</td>
<td>284.52</td>
<td>113.81</td>
<td>1.31</td>
<td>283.19</td>
<td>113.27</td>
<td>3.14</td>
</tr>
<tr>
<td>400 (±15.88)</td>
<td>386.54</td>
<td>96.63</td>
<td>4.1</td>
<td>401.80</td>
<td>100.45</td>
<td>5.72</td>
</tr>
</tbody>
</table>

**Table II. Within- and between-run precision, accuracy and recovery for DEA (n = 5)**

<table>
<thead>
<tr>
<th>C nominal ng/ml</th>
<th>Mean C ng/ml (±SD)</th>
<th>Recovery % (±SD)</th>
<th>CV% (Bias%)</th>
<th>Mean C ng/ml (±SD)</th>
<th>Recovery % (±SD)</th>
<th>CV% (Bias%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 (±0.33)</td>
<td>2.46</td>
<td>98.57 (±13.37)</td>
<td>13.56</td>
<td>2.35</td>
<td>101.45</td>
<td>14.23</td>
</tr>
<tr>
<td>7.5 (±1.26)</td>
<td>8.64</td>
<td>115.33 (±15.08)</td>
<td>14.62 (15.33)</td>
<td>7.64</td>
<td>101.90 (±6.87)</td>
<td>6.74 (1.90)</td>
</tr>
<tr>
<td>125 (±4.82)</td>
<td>133.97</td>
<td>107.17 (±3.45)</td>
<td>3.59 (7.17)</td>
<td>131.29 (±6.19)</td>
<td>105.03 (±4.42)</td>
<td>4.71 (5.03)</td>
</tr>
<tr>
<td>200 (±3.12)</td>
<td>194.14</td>
<td>97.07 (±1.56)</td>
<td>1.61 (2.83)</td>
<td>200.18 (±10.16)</td>
<td>100.09 (±5.08)</td>
<td>5.07 (0.09)</td>
</tr>
</tbody>
</table>
modifying the insulin/glucagon ratio in favor of glucagon, gluconeogenesis from amino acids and lipolysis is induced, the released fatty acids being used to maintain energy cell homeostasis [13]. In this case, AMI and DEA accumulated in the adipose tissue would be mobilized, raising problems both during chronic treatment, by increased plasma levels and toxicity, but also in case of discontinuation of treatment, complete elimination from the body requires a longer period of time.

**Conclusions**
The LC-MS method developed to detect AMI and DEA from rat plasma was sensitive and accurate, with an LLOQ (5 ng/ml for AMI, 2.5 ng/ml for DEA), therefore suitable for pharmacokinetic studies, including low doses of substances originated from the adipose tissue deposits and found in plasma after massive lipolysis.

**Author's contribution**
BEŐ - Conceptualization
GJ - Investigation
SzV - Methodology
AM - Validation
ATV - Writing – original draft

**Conflict of interest**
None to declare

**References**