A HIGH-THROUGHPUT HPLC ASSAY FOR LEVOSIMENDAN IN HUMAN PLASMA WITH ESI-MS/MS DETECTION

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Abstract
A new rapid, sensitive and selective liquid chromatography method coupled with ESI-MS/MS detection was developed and validated for the quantification of levosimendan in human plasma. As a part of the optimization process, two ion sources (electrospray ionization – ESI, and atmospheric pressure chemical ionization – APCI) were tested, in both, negative and positive ion mode and with different mobile phases for the chromatographic separation. The final assay consisted in a separation performed on a Zorbax SB-C18 column (100 mm x 3.0 mm i.d., 3 µm) with isocratic elution using a 66/34 (% v/v) mixture of 0.1% acetic acid /acetoniitrile as mobile phase, followed by a gradient washing step. The MS/MS detection was performed by using ESI in negative ion mode and with m/z = 279.1→227.1 as the final transition monitored. The assay presents several advantages, such as the small sample volume (200 µL), simple and rapid sample processing technique (protein precipitation), high sensitivity and a very good selectivity guaranteed by the MS/MS detection. The method was successfully applied to a pharmacokinetic study in human subjects after an infusion of L-SIM.

Rezumat
S-a elaborat o nouă metodă rapidă, sensibilă și selectivă de dozare a levosimendanului din plasma umană prin cromatografie de lichide cuplată cu detecție ESI-MS/MS. Ca parte a procesului de optimizare s-au testat două surse de ionizare (electrospray ionization – ESI, și atmospheric pressure chemical ionization – APCI), operate atât în modul de ionizare negativă cât și pozitivă, precum și mai multe faze mobile în vederea separării cromatografice. Metoda finală a constat într-o analiză cromatografică pe o coloană Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3 µm) și o fază mobilă formată dintr-un amestec 66/34 (% v/v) acid acetic 0,1%/acetoniitrile, cu eluare în sistem izocratic, urmată de o etapă de spălare în sistem de gradient. Detecția MS/MS a apelat la sursa ESI în modul de ionizare negativă, tranziția finală monitorizată fiind m/z = 279.1→227.1. Metoda elaborată prezintă o serie de avantaje, precum volumul redus de matrice biologică necesar (200 µL), prelucrarea simplă și rapidă a probelor (deproteinizare), precum și sensibilitatea crescută și selectivitatea garantate de cuplarea cu detecția MS/MS. Metoda elaborată s-a aplicat cu succese într-un studiu de farmacocinetica pe subiecți umani după administrarea în perfuzie a levosimendanului.

Keywords: levosimendan, liquid chromatography, mass spectrometry, electrospray ionization, plasma, validation

Introduction
Levosimendan ([R]-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazonodipropenodinitril) is a new calcium-sensitizing and vasodilatory drug developed for the treatment of congestive heart failure.

Simendan is a racemic mixture, with levosimendan (L-SIM) being the pharmacologically active enantiomer. The main mechanism of action of L-SIM consists in the enhancement of the sensitivity of cardiac myofilaments to calcium by binding to troponin C in a calcium-dependent way, resulting in an increased contractile force of the myocardium. The drug has vasodilatory effects also, explained mainly by the opening of an adenosine triphosphate (ATP)-sensitive channel [5, 9] L-SIM offers certain benefits in comparison with traditional inotropes, since it enhances cardiac function without significantly increasing cardiac oxygen consumption or promoting arrhythmia [3, 6].

After entering in the blood circulation, L-SIM is highly bound to plasma proteins (97-98%). The drug is extensively metabolized in man followed by excretion of metabolites in urine and faeces. A minor metabolic pathway is the reduction of L-SIM.
by the intestinal bacteria to OR-1855, followed by rapid acetylation to an active metabolite, OR-1896. The possibility of an enterohepatic circulation of the drug or its metabolites is suggested by the presence of active metabolites even after intravenous infusion [6, 9]. The elimination half-life of L-SIM is approximately 1 h [6]. Steady state concentrations of 14.9 ng/mL and 34.6 ng/mL were reported after a continuous infusion for 7 days of 0.05 µg/kg/min and 0.1 µg/kg/min, respectively [2]. After oral administration (0.5 mg, four times daily for 3 days) a maximum plasmatic concentration of 22.4 ng/mL was reported [1]. The haemodynamic effects of L-SIM seem to continue for several days after treatment cessation. The explanation for this is the very long half-life (approximately 70-80 h) of the metabolites OR-1855 and OR-1896 [2, 9, 12]. To our knowledge there are only few methods published in the literature concerning the determination of L-SIM in biological samples. These assays are based on high-performance liquid chromatography (HPLC), coupled with UV or mass spectrometric detection. There were published few assays concerning chiral separation of the enantiomers of simendan based on capillary electrophoresis (CE) or HPLC, but they focused on the separation and quantification of the enantiomers from racemate solutions and not from biological samples [10, 24]. Wikberg et al. proposed an enantiospecific HPLC-UV method for determination of the ratio of the simendan enantiomers concentrations in rat, dog and human plasma samples. The assay showed a lower limit of quantitation (LLOQ) of 10 ng/mL [23]. Karlsson et al. elaborated a reversed-phase HPLC (RP-HPLC) method with UV detection at 380 nm for quantification of L-SIM in human plasma, after sample preparation involving on-line dialysis and trace enrichment of the dialysates. The total analysis time was approximately 19 min/sample. The lower limit of quantitation (LLOQ) was 5 ng/mL L-SIM [7]. McGough et al. performed liquid-liquid extraction of L-SIM from dog plasma, followed by quantification using a HPLC-UV method (λ = 380 nm). The retention time of L-SIM was of 5.2 min [14]. Antila et al. described a HPLC-MS/MS assay for the simultaneous determination of L-SIM and its metabolites, OR-1855 and OR-1896, in human plasma, with the sample preparation consisting in liquid-liquid extraction. The method showed very good sensitivity with LLOQ of 0.2 ng/mL, 1 ng/mL and 0.2 ng/mL for L-SIM, OR-1855 and OR-1896, respectively [2]. Li et al. reported the quantification of L-SIM and its metabolites in human plasma by HPLC-MS/MS, separately. Since the chemical properties of L-SIM are significantly different from those of its metabolites, two different HPLC methods and different ionization modes were needed (one LC method with MS/MS in negative ion mode for L-SIM, and another method with positive ionization for the metabolites, OR-1855 and OR-1896). For L-SIM the sample preparation consisted in protein precipitation with methanol, while in the case of the metabolites a liquid-liquid extraction was performed. The methods showed good sensitivity, with LLOQ values of 0.1 ng/mL and 0.2 ng/mL for L-SIM and the two metabolites, respectively [11]. Another HPLC-MS/MS method was reported by Zhang et al. A 96-well liquid-liquid extraction procedure was developed for sample preparation. The analysis was performed using sequential negative (for L-SIM) and positive (for the metabolites) ionization LC-MS/MS with a chromatographic run time of approximately 10 min. Two mobile phases and two injections were needed for each sample in order to quantify L-SIM and its metabolites also. This assay needed a special, more complicated instrument configuration. The LLOQ was 0.2 ng/mL for L-SIM and 0.5 ng/mL for both metabolites [25]. The aim of this study was to elaborate and validate a new high-throughput HPLC-MS/MS method for the quantification of L-SIM in human plasma, which can be successfully applied to investigate the pharmacokinetic parameters of L-SIM or potential pharmacokinetic interactions involving the selected drug [26-30].

Materials and Methods

Chemicals and reagents

L-SIM of pharmaceutical purity was obtained from Orion Corporation (Espoo, Finland). Acetonitrile (HPLC gradient grade) and acetic acid (analytical grade) were purchased from Merck (Merck KgaA, Darmstadt, Germany). Purified, type 1 water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. Drug free human plasma (obtained by centrifugation of whole blood, with ethylenediaminetetraacetic acid (EDTA) as anticoagulant) was supplied by the Local Blood Transfusion Centre Cluj-Napoca, Romania.

Calibration standards and quality control samples

A primary stock solution of L-SIM (2.52 mg/mL) was prepared in methanol. Working solutions of L-SIM (10.08 µg/mL and 50.40 ng/mL) were obtained by diluting specific volumes of stock solution with blank plasma. These working solutions were used to spike different volumes of human plasma, providing finally eight standards with the concentrations ranging between 0.50 – 80.64 ng/mL. Quality control (QC) samples containing 1.51, 10.08 and 60.48 ng/mL L-SIM were prepared by diluting specific volumes of L-SIM working standard with blank human plasma.

Chromatographic and mass spectrometry conditions

LC analysis was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump,
Chromatographic separation was achieved on a Zorbax SB-C18 column (100 mm x 3.0 mm i.d., 3 µm) (Agilent Technologies, Palo Alto, CA, USA) preceded by a 0.5 µm online filter. The mobile phase consisted of a 66/34% (v/v) mixture 0.1% acetic acid (solvent A)/acetonitrile (solvent B), with the elution according to the following gradient: 34% solvent B maintained from 0 to 1.80 min, followed by an increase to 90% B at 1.81 min and held at this percentage until 2.40 min, with return to the initial conditions (34% B) at 2.41 min and equilibration of the column for 1.1 min. Each solvent was degassed in an ultrasonic bath before use. The mobile phase was delivered at a flow rate of 1 mL/min. The column temperature was maintained at 50°C.

The HPLC system was coupled to an Agilent MSD VL Ion Trap detector (Bruker Daltonik, GmbH, Bremen, Germany) equipped with an electrospray interface (ESI) operated in the negative ionization mode. Chromatographic and mass spectrometric data acquisition were performed using Chemstation (Agilent Technologies, Palo Alto, CA, USA), version B.01.03 and LC/MSD Trap Control (Bruker Daltonik, GmbH, Bremen, Germany), version 5.3, while data processing was performed using LC/MSD Quant Analysis (Bruker Daltonik, GmbH, Bremen). The mass spectrometer was set for isolation and fragmentation of the deprotonated pseudomolecular ion of L-SIM, with the monitoring of the transition m/z = 279.1 → 227.1. The mass spectrometric parameters were as follows: nebulizer gas, nitrogen at 60 psi; dry gas, nitrogen at 12 L/min; dry gas temperature, 350°C; capillary voltage, 2300V; capillary exit, -52.0V.

Sample preparation

200 µL blank plasma, calibration standards and QC samples were vortex-mixed (Vortex Genie 2, Scientific Industries) for 10 sec. with 600 µL acetonitrile in 1.5 mL polypropylene tubes. The samples were then centrifuged at 12000 rpm for 5 min (204 Sigma centrifuge, Osterode am Harz, Germany). 200 µL of the supernatant was transferred to another 1.5 mL polypropylene tube, diluted with 200 µL 0.1% acetic acid (v/v), vortex-mixed for 10 sec. The diluted supernatant was transferred to an autosampler vial and 25 µL were injected into the HPLC system.

Ion suppression testing

The ion suppression was evaluated by direct infusion (infusion rate 500µL/h) of a solution of L-SIM (10.08 µg/mL) and concomitant injection of blank plasma samples processed according to the protocol described in the “Sample preparation” section.

Method validation

The assay was validated in accordance with the guidance for industry for the bioanalytical method validation [20-22]. Selectivity was checked by comparing six different plasma blanks with the corresponding spiked plasma samples for interference of endogenous compounds with the analyte.

Linearity was studied by analysing in singlicate, the calibration standards at 8 concentration levels in the range of 0.50 – 80.64 ng/mL. The concentration of analyte was determined automatically by the instrument data system using the external standard method. The calibration curve model was determined by the least squares analysis. Linearity was determined by checking five calibration curves on five different working days. The calibration model was accepted if the residuals were within ± 20% at the lower limit of quantification (LLOQ) and within ± 15% at all other calibration levels and at least two-thirds of the calibration standards meet this criterion, including the highest and lowest calibration levels. Regarding the sensitivity of the assay, the LLOQ was set at the lowest calibration level with an accuracy and precision less than 20%.

Precision is defined as coefficient of variation (CV%) and accuracy as relative deviation expressed as percentage error of the calculated value as compared with target added concentrations (true value). The accuracy and intra-run precision were determined by analysis on the same day of five different samples at each QC level. The inter-run accuracy and precision were determined at the same concentrations of L-SIM, but on five different experimental days.

The relative recoveries were analysed at each of the three QC levels and also at the LLOQ, by comparing the response of treated plasma samples with the response of untreated standards in solvent with the same concentration of L-SIM as the plasma QC sample.

In addition, a stability study of L-SIM in human plasma and in final extract was performed as part of the validation process. This study included the evaluation of stability at room temperature for 4 h, post preparative stability (stability of L-SIM in the final extract for 12 h) in the autosampler and freeze-thaw stability after 3 cycles. The stability studies were performed at the three QC levels selected for accuracy and precision studies.

Results and Discussion

There are only few assays for the quantification of L-SIM in plasma samples. Since only one enantiomer of simendan is active, it is important that the analytical methods used for the quantification of L-SIM in biological matrices to be able to distinguish the active enantiomer of simendan from the inactive one. Wikberg et al. found the pure enantiomer, L-SIM, not to isomerize in vivo [23]. As a consequence, in case of the
administration of pharmaceutical dosage forms containing pure L-SIM, it is not necessary to perform chiral separation. The existing methods present certain drawbacks, which justified the aim of the present study, namely to elaborate a high-throughput, sensitive and selective HPLC method with mass spectrometric detection of L-SIM in human plasma. Due to the fact that the methods of Wikberg et al. and Karlsson et al. present poor sensitivity, with LLOQ values of 10 ng/mL and 5 ng/mL, respectively, these assays might be considered less suitable for pharmacokinetic or bioequivalence studies [7, 23]. Another major disadvantage for some of the existing methods (despite their high sensitivity) is the long duration of the analysis, due either to the laborious sample preparation (liquid-liquid extraction [2, 25] or the long chromatographic run time. All the existing methods include the addition of at least one internal standard in order to compensate for variations in recovery. The method described in this paper includes a simple and rapid sample preparation technique, involving protein precipitation with acetonitrile, followed by dilution with 0.1% acetic acid (v/v). The absence of the extraction step reduced the need for an internal standard because in case of protein precipitation there is no partition process of analytes between the two phases (liquid-liquid extraction [2, 25] or the long chromatographic run time. All the existing methods include the addition of at least one internal standard in order to compensate for variations in recovery.

The method described in this paper includes a simple and rapid sample preparation technique, involving protein precipitation with acetonitrile, followed by dilution with 0.1% acetic acid (v/v). The absence of the extraction step reduced the need for an internal standard because in case of protein precipitation there is no partition process of analytes between the two phases (liquid-liquid or liquid-solid phases), making the recovery near 100% and very reproducible (Table II). The sample treatment with acetonitrile destroyed the three-dimensional structure of proteins and allowed the detachment of L-SIM from these endogenous molecules. The high solubility of the analyte in acetonitrile facilitated its transfer into the organic solution, preventing this way the losses that usually might be associated with the protein precipitation step. The construction of the calibration curves and quantification were performed by using the external standard method.

The influence of the sample solvent on the peak shape is well documented in the literature. It is recommended to avoid using a sample solvent with higher elution strength than the mobile phase, because it adversely affects the band profiles leading to poor chromatographic performance. Peak broadening, tailing or even peak splitting can be observed [4, 8, 16]. In case of the method described in this paper the supernatant obtained after deproteinisation had a higher organic content (approximately 75% acetonitrile) than the mobile phase, increasing this way the risk of peak distortions. As a consequence, the supernatant was diluted with aqueous phase, making the sample compatible with the mobile phase.

ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) are the most frequently used ionization techniques used in case of LC coupled with mass spectrometry. During the optimization process, both ion sources were tested with operation in both positive and negative ion mode, and with several mobile phase compositions. The peak area and signal to noise ratio were determined for each combination in order to select the optimum combination of ion source, ionization mode and mobile phase composition. When comparing positive and negative ionization modes, a significant difference was observed with negative ionization showing 80 to 100 fold more intense signal (ionization yield) than the positive mode (results not shown here). The next step in the optimization process focused on tests with both ESI and APCI, operated in negative mode. Several mobile phases were evaluated with the results being summarized in Table I.

Table I
Results obtained during method optimization with ESI and APCI operated in negative ion mode and several mobile phases

<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent A</th>
<th>% A</th>
<th>Solvent B</th>
<th>% B</th>
<th>Ion source</th>
<th>Peak area</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 mM ammonium formate</td>
<td>62</td>
<td>MeOH</td>
<td>38</td>
<td>ESI</td>
<td>1040</td>
<td>151</td>
</tr>
<tr>
<td>2</td>
<td>0.1% formic acid</td>
<td>55</td>
<td>MeOH</td>
<td>45</td>
<td>ESI</td>
<td>443</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>0.1% acetic acid</td>
<td>55</td>
<td>MeOH</td>
<td>45</td>
<td>ESI</td>
<td>1333</td>
<td>140</td>
</tr>
<tr>
<td>4</td>
<td>0.05% ammonium hydroxide</td>
<td>75</td>
<td>MeOH</td>
<td>25</td>
<td>ESI</td>
<td>884</td>
<td>163</td>
</tr>
<tr>
<td>5</td>
<td>0.05% ammonium hydroxide</td>
<td>85</td>
<td>ACN</td>
<td>15</td>
<td>ESI</td>
<td>413</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>0.1% acetic acid</td>
<td>70</td>
<td>ACN</td>
<td>30</td>
<td>ESI</td>
<td>919</td>
<td>174</td>
</tr>
<tr>
<td>7</td>
<td>1 mM ammonium formate</td>
<td>70</td>
<td>ACN</td>
<td>30</td>
<td>ESI</td>
<td>553</td>
<td>123</td>
</tr>
<tr>
<td>8</td>
<td>1 mM ammonium formate</td>
<td>70</td>
<td>ACN</td>
<td>30</td>
<td>APCI</td>
<td>85</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>0.05% ammonium hydroxide</td>
<td>85</td>
<td>ACN</td>
<td>15</td>
<td>APCI</td>
<td>59</td>
<td>38</td>
</tr>
<tr>
<td>10</td>
<td>0.1% acetic acid</td>
<td>70</td>
<td>ACN</td>
<td>30</td>
<td>APCI</td>
<td>49</td>
<td>33</td>
</tr>
<tr>
<td>11</td>
<td>0.1% acetic acid</td>
<td>55</td>
<td>MeOH</td>
<td>45</td>
<td>APCI</td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>1 mM ammonium formate</td>
<td>62</td>
<td>MeOH</td>
<td>38</td>
<td>APCI</td>
<td>38</td>
<td>21</td>
</tr>
</tbody>
</table>

Solvent A - Aqueous phase; Solvent B – Organic phase; S/N – signal to noise ratio; MeOH – methanol; ACN - acetonitrile
Different ion sources are recommended in case of different types of analytes. The APCI source is mostly recommended in case of relatively non-polar analytes which are able to ionize in the gas phase, while ESI is more suitable for analytes capable of ionization in solution [15, 17]. The results obtained during the optimization process showed superior efficacy of the electrospray ionization source (Table I). The highest signal intensity and S/N ratio was obtained with acetic acid as the mobile phase additive combined with acetonitrile. The final mass spectrometric conditions consisted in electrospray ionization source operated in negative ion mode, with the isolation and further fragmentation of the deprotonated pseudomolecular ion (m/z = 279.1). The final transition monitored was m/z = 279.1→227.1 (Figure 1). The optimum mobile phase used for the chromatographic separation consisted in a mixture of 0.1% acetic acid/acetonitrile.

Selecting an acid mobile phase was justified also by the pKₐ value reported in the literature for L-SIM (pKₐ = 6.3) [10]. An acidic mobile phase guaranteed the presence of the analyte in non-ionized form, having this way a higher affinity and adequate retention on the non-polar reversed phase chromatographic column (stationary phase) (Figure 2). In order to maintain a single form of the analyte, the aqueous phase used for the dilution of the supernatant before injection contained also an acidic reagent (0.1% (v/v) acetic acid).

Representative extracted ion chromatograms of blank plasma (a), same blank plasma spiked with L-SIM at LLOQ level (0.504 ng/mL) (b) and a real human plasma sample collected at 40 min after starting the L-SIM infusion (c)

An important issue in LC analysis coupled with mass spectrometry is the influence of residual compounds present after the sample preparation process, leading to a phenomenon termed matrix effect. One possible explanation of the matrix effect is the ionization competition between the different species present in the effluent, leading to ion suppression. The existing data regarding the matrix effect indicates ESI as being more susceptible to matrix effect than APCI, because in case of APCI ionization takes place in the gas phase [13, 18, 19]. The matrix effect study showed important ionization suppression between 0.35 - 1.2 min, but there were no significant changes in signal intensity between 1.65 - 1.85 min (Figure 3), a time interval including the retention time of L-SIM (1.7 min).

The ion suppression observed during the chromatographic run – injection of a blank plasma sample and concomitant infusion (infusion rate 500µL/h) of a L-SIM standard solution (10.08 µg/mL)
The optimum mobile phase used for the chromatographic separation consisted in a mixture of 0.1% acetic acid/acetonitrile. The elution of the analyte could be performed under isocratic conditions (between 0 - 1.80 min), but in the second part of the chromatographic run a washing step with gradient elution was introduced in order to elute some endogenous compounds, more hydrophobic and more strongly retained than the analyte. Without this washing step it is possible that these late eluting compounds to induce ion suppression after a great number of injections.

Using the final chromatographic conditions and the ESI source operated in negative ion mode, the method was validated in accordance to the industrial guidance for the bioanalytical method validation in terms of selectivity, sensitivity, linearity, accuracy and precision. The analysis of blank samples showed no significant interferences at the retention time of L-SIM (Figure 3). Standards of the metabolites of L-SIM were not available for analysis, however since they are presenting different molecular masses than the parent compound; it can be considered that the MS/MS detection guarantees the lack of any interferences from their part in the selected analytical conditions. The calibration curves showed good linearity over the studied concentration range (0.504 – 80.64 ng/mL). The method showed similar or even superior sensitivity to methods described in other scientific papers, based on LC analysis and a more laborious sample preparation or longer chromatographic run time. The LLOQ was set at the level of the lowest calibrator (0.504 ng/mL), with accuracy and precision less than 20% (Table II). The within-run precision and accuracy data are summarized in Table II. According to these results the assay is accurate and precise over the studied concentration range.

### Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>c_{nominal} (ng/mL)</th>
<th>Mean c_{found} (ng/mL) ± SD</th>
<th>CV%</th>
<th>Inaccuracy%</th>
<th>Recovery% ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.504</td>
<td>0.47 ± 0.04</td>
<td>9.2</td>
<td>-7.0</td>
<td>102.0 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>1.512</td>
<td>1.37 ± 0.02</td>
<td>1.5</td>
<td>-9.2</td>
<td>104.7 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>10.080</td>
<td>10.98 ± 0.46</td>
<td>4.2</td>
<td>8.9</td>
<td>98.0 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>60.480</td>
<td>59.36 ± 2.47</td>
<td>4.2</td>
<td>-1.9</td>
<td>102.3 ± 8.5</td>
<td></td>
</tr>
<tr>
<td><strong>Between-run</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.504</td>
<td>0.55 ± 0.04</td>
<td>7.2</td>
<td>9.6</td>
<td>100.2 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>1.512</td>
<td>1.53 ± 0.16</td>
<td>10.4</td>
<td>1.3</td>
<td>101.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>10.080</td>
<td>10.99 ± 0.33</td>
<td>3.0</td>
<td>9.0</td>
<td>97.2 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>60.480</td>
<td>55.95 ± 3.17</td>
<td>5.7</td>
<td>-7.5</td>
<td>95.5 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

\[c_{\text{nominal}} - \text{nominal concentration; } c_{\text{found}} - \text{found (measured) concentration; } \text{SD} - \text{standard deviation; } \text{CV} - \text{coefficient of variation}\]

The results of the stability study revealed no significant changes in L-SIM concentration under the evaluated conditions.

The elaborated method was applied in a study on real human plasma samples (obtained by using the same anticoagulant (EDTA)), to evaluate the pharmacokinetic parameters of L-SIM in patients undergoing cardiac surgery. A chromatogram corresponding to a real human plasma sample after L-SIM infusion is presented in Figure 3(c), corresponding to a concentration of 35.95 ng/mL.

### Conclusions

This article describes a high-throughput analytical method for the quantification of L-SIM in human plasma. During the optimization process two ion sources (ESI and APCI) were tested in both, positive and negative ion mode with ESI operated in negative ion mode showing much higher efficiency of the ionization process. After selecting the optimum mobile phase and MS/MS detection parameters, the method was validated in accordance with the bioanalytical methods validation guidelines and showed good linearity, accuracy and precision in the studied concentration range. The major advantages of this assay are the small sample volume (200 µL), simple and rapid sample processing technique (deproteinization), high sensitivity and a very good selectivity guaranteed by the MS/MS detection. The method was successfully applied to a pharmacokinetic study of L-SIM in human subjects after an infusion of L-SIM. All the analytical and economic advantages of the assay together with its application on real human samples recommend this method as being adequate for pharmacokinetic and bioequivalence purposes.

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