Ultra-Performance Liquid Chromatography – Tandem Mass Spectrometric Determination of an Antihypertensive Drug in Human Plasma

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Conflict of Interests

No conflict of interest associated with this work.

Contribution of Authors

I declare that this work was done by the author named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the author.
ABSTRACT

Purpose
Ramipril (RAM) is a drug used to control blood pressure and other associated disorders. Different analytical methods were adopted for its quantification but there is an urgent and continuous need to adopt new methods for more sensitive determination of the analyte with smaller lower quantification limit and also with wider linearity range.

Method
The current study introduces a liquid chromatographic method coupled with tandem mass spectrometry for the quantification of RAM in biological fluids (plasma). The samples were subjected to liquid extraction using n-hexane: propanol (1:1, v/v). In this work, an internal standard was used which is atorvastatin. RAM was up taken from the biological fluid using n-hexane: propanol (1:1, v/v), then chromatographed on a suitable C₁₈ column with a mobile phase composed of methanol: water with 0.1% formic acid (4: 1, v/v) and the flow rate was 0.2 mL/min.

Results
RAM is determined in a highly sensitive way even in biological fluids, where it can be easily and simply quantified in a range of 0.05 - 1000 ng/mL. The validation procedure for the proposed method was carried out including sensitivity, selectivity, stability and study of the matrix effect.

Conclusion
The outcomes of the work showed that, it can be used for the estimation of the studied drug for bioavailability and pharmacokinetic studies.

Keywords: Liquid chromatography–tandem mass, Ramipril, Stability, Biological fluids, Plasma.
1. Introduction

Hypertension is a serious condition associated with an elevation in blood pressure. It may be known as the silent killer as in some cases, it has no clear symptoms. Uncontrolled persistent high blood pressure may lead to disastrous diseases like heart failure, stroke, atrial fibrillation and kidney male function [1]. RAM is \((2S,3aS,6aS)-1-\{N-[(S)-1-ethoxycarbonyl-3-phenyl-propyl]l-alanyl\}perhydrocyclopenta[b]pyrrole-2-carboxylic acid\). It can be considered as a prodrug, where it needs to be activated in liver by bio-transformation into the ramiprilate form (active form). RAM exhibits its action as antihypertensive drug via its effect on angiotensin converting enzyme by its inhibition and so preventing the conversion of angiotensin I into angiotensin II, which has a vasopressor action on the smooth muscles of the arteries leading to arterial vasodilatation. The studied drug is used as an antihypertensive agent that reduces the risk of heart failure. In addition, it can reduce the risk of heart attack and stroke [2].

On reviewing the quantification procedures concerning RAM in biological fluids, many analytical techniques were applied for its determination in the biological samples including liquid chromatography coupled with tandem mass detection either alone or in combination with other therapeutic agents [3-8], high performance liquid chromatography (HPLC) [9] and spectrophotometry [10].

LC-MS/MS has a significant advantage in selectivity and sensitivity. The high selectivity leads to advantageous separation pattern and sharper peak outline that leads to improvement in method sensitivity and reduction in the time needed for analysis [11].

Regarding the previously published work on RAM, they have acceptable sensitivity and linearity ranges, but the aim of this work is to introduce a liquid chromatographic method coupled with tandem mass detection with better sensitivity and wider linearity range than the previously published work. Also, the present work aims to give a complete validation procedure for the proposed method. In the same time this work wants to introduce a simple analytical method with short analysis time. This work is designed to give a tool to quantify RAM in plasma during the pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

RAM was supplied by Sanofi Aventis Pharma, Egypt; its purity was documented as 100.4%. Atorvastatin was supplied by Aqa international Pharmaceuticals, India. Its purity was documented as 99.9%. Human plasma was
obtained from King Khaled hospital, Al-Kharj, KSA. Methanol, n-hexane, formic acid and propanol were purchased from Sigma Aldrich. HPLC grade water was obtained from Merck, Germany.

2.2. Instrumentation

Waters Acquity™ liquid chromatograph working with a quaternary pump and autosampler. It is equipped with a tandem mass detector of triple quadrupole type.

2.3. Chromatographic and mass conditions

Chromatographic analysis was performed using Waters Acquity™ chromatograph with a column of RP-C<sub>18</sub> (15 cm x 2.1 mm, 1.7 µm particle size). The composition of developing system was methanol: water with 0.1% formic acid (9: 1, v/v). Degassing of the developing system was done using in a sonicator for 15 minutes. The optimum used flow rate was 0.2 mL/min. at room temperature.

Operation of the tandem mass spectrometer was carried out using multiple reactions monitoring. The positive ionization mode was applied for the analysis procedure. The optimized mass spectrometric conditions are introduced in Table 1.

2.4. Standard and quality control samples preparation

2.4.1. Stock and working standard solutions

RAM and IS stock standard solutions (100 µg/mL) were separately prepared using methanol as a solvent. All solutions were stored at 4–8 °C. The working calibration solutions and controls were prepared by suitable dilutions using methanol as a diluting solvent. The IS working solution (5 µg/mL) was prepared by the appropriate dilution of its stock standard solution using methanol as a diluting solvent. Working solutions for calibration were separately prepared in the range of 0.5–10000 ng/mL via dilution of specific volumes of the stock standard solution using methanol as a diluting solvent.

2.4.2. Quality control samples and calibration standards preparation

Suitable portions of RAM working standard solutions were added to spike 350 µL blank plasma to prepare seven plasma standards in a concentration range of 0.05-1000 ng/mL. Quality control (QC) samples having concentrations of 0.05 ng/mL (lower limit of quantification LLOQ), 0.3 ng/mL (QCL), 400 ng/mL (QCM) and 800 ng/mL (QCH) were utilized during the validation procedure.
2.5. *Extraction procedure*

Fifty microliters of the IS working standard solution was transferred to the spiked plasma with RAM. Vortex stirring of the resulted solution for 20 seconds was done. After that 5 mL of the mixture used for extraction (n-hexane: Propanol, 1:1, v/v) was added. The resulted mixture was vortex mixed for 60 seconds. Centrifugation of the mixture for 4 minutes was carried out. Transferring of 4 mL of the organic layer to centrifuge tubes was done followed by evaporation at 65°C using a vacuum concentrator. Reconstitution by adding 200 µL of the mobile phase was performed. Finally, 10 µL of the sample was then introduced into the chromatographic system.

2.6. *Validation Protocol*

Validation of the analytical procedure is a very important step in method optimization and this task can be fulfilled according to the United States Food and Drug Administration (FDA) guidelines [12].

2.6.1. **Selectivity**

The probability of interference from endogenous substances in human plasma with the quantification procedure concerning RAM or IS was assessed by chromatographing blank plasma samples before any analyte analysis.

2.6.2. **Linearity**

Linearity was assessed by constructing calibration graphs utilizing seven samples in the range of 0.05 - 1000 ng/mL RAM. Also, a blank plasma sample containing the IS was chromatographed to ensure the lack of any interference. The calibration graphs were plotted using the ratio between the RAM to IS peak area in different days.

2.6.3. **Lower limit of quantification (LLOQ)**

Analysis of seven replicates of plasma samples containing the LLOQ to assess the sensitivity of the proposed method.

2.6.4. **Accuracy and precision**

Accuracy and interday precision were assessed through the manipulation of five samples of each quality control concentration (in the linearity range) at different days. On the other hand, the intraday precision and accuracy were assessed by analyzing five samples of each quality control concentration within the linearity range in the same day. Judging the precision was done via calculating the % CV. The value of % CV should not exceed a value of 15%, but there is an exception for LLOQ, where it can be about 20%.
2.6.5. Matrix effect and sample recovery

RAM recovery was assessed by comparing the area of peak for three different concentrations within the linearity range in the pre-extraction samples (These are plasma samples spiked with the studied drug and subjected to the whole extraction process), with their peak areas in the post extraction samples (These are plasma samples spiked with the studied drug at the end of the extraction procedure) [13].

The matrix effect was assessed by handling blank plasma samples from five sources with RAM using the QCM. The matrix effect can be expressed by calculating the ratio between the area under the peak of the studied drug in the post extraction samples to the peak area of the methanolic standard solutions at equivalent concentrations. If the calculated ratio equals to unity, this may indicate no matrix effect. On the other hand, if the ratio exceeds the value of unity, this may indicate ionization enhancement. A fraction value may suggest suppression of ionization.

Carryover with respect to RAM and the IS is tested by carryover test. The samples of the carryover test were analyzed as the following pattern; blank plasma sample, the upper quantification limit (ULOQ) sample and finally, the blank plasma sample.

2.6.6. RAM stability

It could be evaluated in the room temperature conditions as short term stability. Post-preparative stability can be assessed in the autosampler (25°C). Also, freeze–thaw stability and long-term stability were checked at −85 °C. In all stability procedures, QCM, QCL and QCH were analyzed three times for each QC sample. The QC samples were freshly manipulated and chromatographed in a single run to act as time zero (fresh samples).

2.6.6.1. Short term stability

All samples were left at room temperature for four hours, which is a very sufficient time for the preparation of the sample. After 4 hours, a standard curve was plotted in a single run using all stability samples. The stability samples were compared to the fresh samples at equivalent concentration.

2.6.6.2. Post preparative stability

Three QC samples were manipulated then left in the auto sampler which is thermostated at 25 °C. The prepared samples were analyzed after 12 hours.
2.6.6.3. Freeze and thaw stability

It was evaluated by subjecting the plasma samples to 3 cycles of freeze–thaw at −85 °C during the day in 3 consecutive days. Analysis of the samples was done after the third cycle and calculating their concentrations was carried out in the same day. Comparison of equal concentrations from the fresh and test samples were done to assess the analyte stability.

2.6.6.4. Stability as a long-term issue

Storage of three replicates of each quality control sample was done −85 °C for thirty days. Following the storage stability definition by comparing sample concentration to the mean concentration values calculated at the day one of the analysis. Calculation of the values was done using a standard curve prepared at the same day then comparing the concentration of stored samples with that of the fresh ones.

3. Results and Discussion

The goal of this effort is to establish and validate an analytical method can be applied for the sensitive and reliable quantification of RAM in plasm samples and extending this procedure to be applied for its determination during the pharmacokinetic studies. The enormous sensitivity and selectivity of tandem mass spectrometric detection, it is coupled with liquid chromatography in this work to establish a more sensitive analytical method than the already published work with a wider linearity range and so this will make a great benefit for the ease application of this method in the pharmacokinetic studies.

3.1. Optimization of the mass spectrometric conditions

Positive ion mode scanning for the studied drug and IS was used to get full mass scan. The most sensitive mass transitions were from m/z 416.97 / 234.19 and 559.39 / 440.27 for RAM and IS, respectively. Method development was carried out using the MRM mode to get the maximum selectivity and sensitivity for the propose method. The working MRM parameters were optimized and given in Table 1.
Table 1. Main working parameters of the tandem mass spectrometer.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ramipril (°C)</th>
<th>IS (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source temperature (°C)</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Dwell time per transition (second)</td>
<td>0.063</td>
<td>0.146</td>
</tr>
<tr>
<td>Capillary (kV)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Desolvation temperature (°C)</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Desolvation gas flow (L/Hr)</td>
<td>800 L/hr</td>
<td>800 L/hr</td>
</tr>
<tr>
<td>Cone (V)</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Collision energy (V)</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Mode of analysis</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Ion transition (Da) m/z</td>
<td>416.97/234.19</td>
<td>559.39/440.27</td>
</tr>
</tbody>
</table>

3.2. Sample preparation procedure and chromatographic condition optimization

Sample preparation should be simple, fast and can be accomplished with small reagent quantities and reproducible acceptable drug recovery. According to the literature, there are several methods for sample preparation and drug extraction [14,15]. In this work, liquid–liquid extraction was used to get a small time for sample preparation if compared with solid phase extraction and also to get reproducible and acceptable drug recovery. Many solvents were tried for the process of extraction to get the maximum recovery which in turn will be reflected on the sensitivity of the proposed analytical method. Maximum recovery and optimum sample preparation time were attained using n-hexane: propanol (1:1, v/v) as a solvent for extraction of the analyte from the plasma samples without the need to adjust pH. The endogenous plasma matrix shows no interference. Also, IS did not change the analyte sensitivity and/or recovery.

The chromatographic conditions were optimized in order to get the best shape of the analyte peak and the highest sensitivity. Different stationary phase/ mobile phase match was tried to get the optimum separation and highest sensitivity. This task was accomplished by using Waters™ UPLC with a reversed phase C18 column (15 cm
x 2.1 mm, 1.7 µm particle sizes). The mobile phase was methanol: water containing 0.1% formic acid (4: 1, v/v), where the best sensitivity was attained on using this mobile phase.

Atorvastatin was used as an internal standard as it was recovered successfully on using the cited extraction protocol. Also, it was eluted with a reasonable retention behavior on applying the optimized chromatographic conditions.

A short analysis time of three minutes was established on applying the optimized chromatographic conditions, where the average retention times were 1.7, 2.0 min for RAM and IS, respectively.

3.3. Method performance and validation

3.3.1. Selectivity

The wonderful method selectivity is due to its capability of monitoring the drug fragments obtained from the parent analyte ion. Method selectivity was checked by chromatographing the extract of human plasma samples which act as blank experiment (Fig.1), where there is no significant interferences coming from the endogenous materials in the human plasma free from the analyte.

![Fig.1 A chromatogram representing blank plasma samples.](image)

3.3.2. Quantification lower limit and linearity

The excellent sensitivity was evaluated by calculating signal to noise (S/N) ratio. It was found to be 30 and 299 for the studied drug and IS, respectively. According to the LLOQ, the analyte can be successfully quantified in human plasma samples with up to 0.05 ng/mL. The values of LLOQ enables the RAM quantification in plasma samples after swallowing of a dose of all RAM pharmaceutical preparations.
The standard curve was linear the range of 0.05 - 1000 ng/mL. The curve linearity was assessed by correlation coefficient (r) calculation and by back calculating the concentrations of the calibration standards.

The linear regression equation was computed to be: \( y = 0.005 \pm 0.00007 \times + 0.0324 \pm 0.001 \), taking into consideration that, \( y \) is the peak area ratio of RAM to the IS. On the other hand, \( X \) is the concentration of RAM. The standard curve average correlation coefficient obtained during the validation process was 0.9979 ± 0.002. The LLOQ was found to be 0.05 ng/mL, in human plasma with an accuracy of 105.00%. Precision (CV%) was found to be 15.43. Figure 2 introduces LLOQ chromatograms.

3.3.3. Assessment of accuracy and precision

The accuracy and precision, either the inter-day or the intra-day precision, for the studied drug in quality control samples were presented in Table 2.
Table 2. Precision and accuracy for RAM determination.

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Mean recovery ± RSD%*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
</tr>
<tr>
<td>QCL</td>
<td>104.24±13.62</td>
</tr>
<tr>
<td>QCM</td>
<td>97.63±7.10</td>
</tr>
<tr>
<td>QCH</td>
<td>98.75±4.53</td>
</tr>
</tbody>
</table>

* Mean percentage recovery and RSD% were calculated using six determinations

3.3.4. Assessment of the matrix effect and recovery

A matrix effect of 0.83 was acquired for QCM and so, minor suppression of ionization was thought but it did not affect any of the validation parameters.

RAM and IS recoveries were assessed as mentioned in the experimental part. The mean RAM recovery from spiked plasma samples was 88.74±2.97%. The internal standard recovery was 87.22±0.97%.

3.3.5. Carryover test

Carryover was examined to be sure that there is no effect from neither RAM nor the IS on upcoming injections. Concerning this work, there is no carryover effect was observed.

3.3.6. Stability assessment

RAM stability was assured at room temperature for four hours, which is the maximum sample preparation time. There is no observed degradation when the manipulated samples were stored in the auto-sampler at 25°C for twelve hours. Also, no observed variation in quality control samples concentration when compared to the freshly prepared ones after 3 cycles of freeze/thaw. Long term stability of the studied drug was also studied at a temperature of −85°C for thirty days (Table 3). Stock solution stability was examined at 8°C for 7 days. The percentage recoveries were 99.14 and 97.84 for RAM and IS, respectively. This may indicate acceptable stability during a week. Daily
preparation of the working solutions was done just before spiking for both the calibration curve and the quality control samples.

Table 3. Stability data under different conditions.

<table>
<thead>
<tr>
<th>Item</th>
<th>% Deviation *from fresh sample concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short term stability (4h at room temp)</strong></td>
<td></td>
</tr>
<tr>
<td>QCH</td>
<td>-0.95</td>
</tr>
<tr>
<td>QCM</td>
<td>1.45</td>
</tr>
<tr>
<td>QCH</td>
<td>7.94</td>
</tr>
<tr>
<td><strong>Post preparative stability (12 h at 25°C)</strong></td>
<td></td>
</tr>
<tr>
<td>QCH</td>
<td>-3.56</td>
</tr>
<tr>
<td>QCM</td>
<td>4.72</td>
</tr>
<tr>
<td>QCH</td>
<td>7.65</td>
</tr>
<tr>
<td><strong>Long term stability (-20°C for 30 days)</strong></td>
<td></td>
</tr>
<tr>
<td>QCH</td>
<td>-4.96</td>
</tr>
<tr>
<td>QCM</td>
<td>5.44</td>
</tr>
<tr>
<td>QCH</td>
<td>-4.32</td>
</tr>
<tr>
<td><strong>Freeze- thaw cycles (three cycles)</strong></td>
<td></td>
</tr>
<tr>
<td>QCH</td>
<td>-3.31</td>
</tr>
<tr>
<td>QCM</td>
<td>3.92</td>
</tr>
<tr>
<td>QCH</td>
<td>1.43</td>
</tr>
</tbody>
</table>

*% deviation = 100 x (Stability sample – Fresh sample/ Fresh sample)*
4. Conclusion

A liquid chromatographic method coupled with tandem mass spectrometric detection was applied for the highly sensitive and selective determination of the widely prescribed antihypertensive agent (RAM). This work is extrapolated for the sensitive and selective determination of the studied drug in human plasma on using atorvastatin as an internal standard. This work has the advantages of lower quantification limits and wider linearity range than the already published work. Moreover, this work has the merits of reproducible sample recovery coupled with minimum matrix effect and simple sample preparation. Also, this method can be applied for the sensitive and selective monitoring of RAM in plasma during the pharmacokinetic studies.

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References


