Method Development, Validation and Pharmacokinetics for Ramipril and Hydrochlorothiazide in Human Plasma and Application in a Bioequivalence Study Based on Healthy Jordanian Volunteers

Ayoub Alhaji1*, Omar Alnasr1, Mahmoud Alawi1*, Tawfiq Arafat1
1 Jordan Center for Pharmaceutical Research, P.O. Box 950435, Amman 11105, Jordan
2 University of Jordan, Chemistry Department, P.O. Box 13003, Amman 11942, Jordan
3 University of Petra, Faculty of Pharmacy, Amman, Jordan

ABSTRACT:
A simple liquid chromatography mass spectrometric method was developed for quantitative determination of Ramipril (RAM) and Hydrochlorothiazide (HCTZ) simultaneously in human plasma. RAM, HCTZ and the internal standards (RAM-D5, HCTZ-13C, D2) were extracted from human plasma by direct protein precipitation. The chromatographic separation was achieved using ACE 5 C8, (50 mm x 4.6 mm, 5µm) column with gradient elution mobile phase of 0.25 mM Ammonium chloride and methanol. The method validation intends to investigate sensitivity, linearity, precision and accuracy, recovery, matrix effect, specificity and stability according to the European Medicines Agency (EMA) guideline. Standard calibration levels were prepared in pooled human plasma to attain final dynamic range of 0.2 - 20.0 ng/mL for RAM and 1.0 - 150 ng/mL for HCTZ. Clinical bioequivalence study was successfully investigated by application of this validated bioanalytical method in order to evaluate bioequivalence of two commercial products for 5mg RAM/ 25mg Hydrochlorothiazide single dose.

INTRODUCTION:
Ramipril
Ramipril (RAM) (Fig.1A), is chemically described as 2-[[N-[[(S)-1-(ethoxycarbonyl)-3-phenylpropyl)]-L-alanyl]-1S,3S,5S]-2-azabicyclo[3-3-0] octane carboxylic acid. RAM is in a group of drugs called angiotensin converting enzyme (ACE) inhibitors. RAM is used to treat high blood pressure (hypertension) or congestive heart failure, and to improve survival after a heart attack [1].

Hydrochlorothiazide
Hydrochlorothiazide (HCTZ) (Fig.1B), is chemically described as 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-Sulphonamide-1,1-dioxide [2]. Hydrochlorothiazide, 3,4-dihydro derivative of chlorothiazide, is a diuretic and antihypertensive drug of the thiazide class that acts by inhibiting the kidneys' ability to retain water. This reduces the volume of the blood, decreasing blood return to the heart [3].

Tritace® Comp
Tritace comp is a combination drug of RAM - a strong and long-acting ACE inhibitor and hydrochlorothiazide - thiazides group of drugs, which promotes fluid excretion (diuretics). A combination of hydrochlorothiazide and RAM in tablet form or capsules is widely used because in certain cases, a single (ACE) inhibitor drug does not respond sufficiently to reduce hypertension, then a combined dosage form is used with other specific classes of drug compounds such as diuretics to moderate the severe hypertension which is not controlled by a single antihypertensive agent and also in older patients who have low Renin levels [4].

RAM and HCTZ has been determined in human plasma by separate bioanalytical methods, which is considered to be costly, time consuming and multiple steps procedure - like liquid-liquid extraction and spectroscopy - required large plasma volume to be divided into two analytical volumes and two different analytical systems or more are needed to determine the active ingredients [3, 5, 6].

To the best of our knowledge, only two methods have been published for simultaneously quantification of HCT and RAM in human plasma. The first method using triple stage quadrupole mass spectrometer with Solid phase extraction and the lower limit of quantification (LLOQ) was 0.5 ng/mL for RAM and 1.0 ng/mL for HCT [7]. The second method using liquid chromatography tandem mass spectroscopy using liquid-liquid extraction procedure and the LLOQ was 2.0 ng/mL for RAM and 8.0 ng/mL for HCT [8]. The present work describes a fast, selective and highly sensitive direct precipitation method, which enables simul-

*Corresponding author:
Mahmoud Alawi
University of Jordan, Chemistry Department, P.O. Box 13003, Amman 11942, Jordan.
E-mail: alawima@ju.edu.jo
Contact: 00962777483679,
Fax: 00962 6 5300253

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taneous determination of RAM and HCT at 0.2 ng/mL for RAM and 1.0 ng/mL for HCT in plasma with good accuracy using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS).

**Experimental**

**Internal standards**

RAM-D5, (Fig.1C) a labeled version of RAM (Fig. 1A) where symbol (D) refers to deuterium and hydrochlorothiazide-13C,D2 (Fig. 1D), a labeled inhibitor of carbonic anhydrase (Fig. 1B) which their retention behaviors are similar to those of the target analytes, were used as internal standards [9, 10].

**Figure 1:** Chemical structure of (A) RAM, (B) HCTZ, (C) RAM-D5 and (D) HCTZ-13C,D2

**Instrumentation**

HPLC system (1200 Series Agilent Technologies, Germany) was used in connection with an Atmospheric Pressure Ionization (API) 3000 mass spectrometer (Applied Biosystems/MDS SCIEX, Canada) which was equipped with an ESI source. Data processing was performed with the Analyst 1.6 software using 1/x linear weighted function for result determination.

**Mass spectrometric detection**

Mass parameters were optimized using the multiple reaction mode (MRM) scan in the positive ionization mode at m/z: 417.1→234.2 for RAM and at m/z: 422.1→239.2 for RAM-D5 with nebulizer gas of 12, curtain gas of 6, turbo ion spray voltage 5500 V, ion source temperature 550°C, collision energy (CE) of 31 eV, collision cell exit potential (CXP) of 12 V, declustering potential (DP) of 71 V, focusing potential (FP) of 270 V, entrance potential (EP) of 10 V and in the negative ionization mode at m/z: 295.9→268.5 for HCTZ and at m/z: 298.9→271.5 for HCTZ-13C,D2 with nebulizer gas of 12, curtain gas of 9, turbo ion spray voltage -1500 V, ion source temperature 550°C, collision energy (CE) of -28 eV, collision cell exit potential (CXP) of -25 V, declustering potential (DP) of -56 V, focusing potential (FP) of -160 V, entrance potential (EP) of -10 V.

**Chromatographic conditions**

Chromatography was performed on an ACE C8 (50 mm x 4.6 mm, 5µm) analytical column with a mobile phase consisting of 0.25 mM ammonium chloride (pH=3.6): methanol using gradient elution system at a flow rates of 0.60 and 0.80 mL/min as shown in table (1). The auto sampler tray temperature was set at 10°C and the injection volume was 5 µl.

**Chemicals and reagents**

HCTZ (purity 99.8 %) was obtained from JOSWE medical (Amman, Jordan), RAM (purity 99.4 %) was obtained from Parmacare PLC (Ramallah, Palestine), HCTZ-13C,D2 Internal Standard (IS) (purity 99.00 %) was obtained from Toronto Research Chemicals (Toronto, Canada), RAM-D5 (IS) (purity 99.00 %) was obtained from Toronto Research Chemicals (Toronto, Canada).

The blank plasma sample was harvested from donors and collected by the blood bank, Islamic Hospital (Amman, Jordan). Plasma was obtained by centrifugation of whole blood samples treated with sodium heparin.

**LC/MS quality (Lichrosolv®) acetonitrile, deionized water and methanol were purchased from (Merck & Fisher, Germany), Acetic acid (99.7 %) (Acros, Germany), Ammonium chloride (99.00 %) (Riedel-deHaën/Germany).**

**Standard solutions**

Stock standard solutions of 1.0 mg/mL for RAM, RAM-D5, HCTZ and 2.5 mg/mL for HCTZ-13C,D2 were prepared in methanol and stored in refrigerator (4-8°C). These solutions were further diluted in (50:50%, v/v, deionized water/ Methanol), to give appropriate working standard solutions used to prepare the calibration curves and to do quality control tests.

**Preparation of standards and quality control (QC) samples**

Standard stock solutions of 1.0 mg/mL for RAM, RAM-D5, HCTZ and 2.5 mg/mL for HCTZ-13C,D2 were prepared separately in methanol. The internal standard spiking solution (5.0 ng/mL of RAM-D5 and 500.0 ng/ml of HCTZ-13C,D2) was prepared in methanol from standard stock solutions of internal standards. Standard stock solutions and internal standard spiking solutions were stored in refrigerator conditions (2–8°C) until analysis. Standard stock solutions of RAM and HCTZ were added to analytes-free human plasma to obtain the final concentrations of 0.2, 0.4, 1.0, 3.0, 5.0, 8.0, 12.0 and 20.0 ng/mL for analytical calibration curve of RAM and concentrations of 1.0, 2.0, 5.0, 10.0, 30.0, 60.0, 90.0 and 150.0 for analytical calibration curve of HCTZ. Similarly, quality control samples were prepared in pooled plasma at the concentrations of 0.2, 0.6, 10.0, 16.0 ng/mL for RAM and 1.0, 3.0, 75.0 120.0 for HCTZ. All the calibration plasma samples were divided into aliquots and stored in deep freezer at −30°C until analysis.

**Sample preparation and method of extraction**

Protein direct precipitation was used to extract the analytes and internal standards from human plasma and for this purpose 200 µL of plasma sample was taken, 400 µL of methanol containing (5.0 ng/mL of RAM-D5 and 500.0
ng/ml of HCTZ-$^{13}$C, D2) were added, vortexed vigorously for 1.0 min then centrifuged for 15 min at 14000 rpm. The supernatant was transferred to an evaporation glass tube and evaporated using a gentle stream of nitrogen at 45°C water bath. The dried residue were reconstituted with 200 μl of (80:20%, v/v Water: Methanol + 1% acetic acid) and vortexed for 1.0 min. The extracted sample was transferred into auto sampler vial and injected into LC-MS/MS.

Bioanalytical Method validation

This method for quantification of RAM and HCTZ in human plasma was validated in accordance with the European Medicines Agency (EMA) guideline taken in consideration the United States FDA guideline requirements. The Validation was performed in order to evaluate the method in terms of linearity of response, accuracy, precision, recovery, sensitivity, specificity, matrix effect and stability [11, 12].

Result and discussion

Method development

Chromatographic conditions, especially the composition and nature of the mobile phase, usage of different columns, different extraction methods such as Precipitation and Liquid-liquid extraction methods were optimized through several trials to achieve the best resolution, highest peak response and short run time for RAM, HCTZ and the internal standards.

Direct precipitation by methanol and evaporation of the supernatant was used for the sample preparation in this work. Direct precipitation can't be helpful in producing a clean sample as LLE and it causes precipitation of matrix materials on curtain plate of the mass spectrometer [13]. To solve this problem we developed a gradient elution system firstly, to get good separation between HCTZ in negative mode and RAM in positive mode which was difficult to achieve by using an isocratic mobile phase. Secondly, to get rid of the sample bulk solution in the first step of the gradient system as it illustrated in table (1), good separation and elution were achieved using a mobile phase of 0.25 mM ammonium chloride buffer (pH=3.6), mixing with methanol.

Table 1: Mobile phase gradient elution - system for RAM and HCTZ

<table>
<thead>
<tr>
<th>Step</th>
<th>Total time(min)</th>
<th>Flow rate (µl/min)</th>
<th>A (%) 0.25 mM Ammonium chloride</th>
<th>B (%) Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>800</td>
<td>85.0</td>
<td>15.0</td>
</tr>
<tr>
<td>1</td>
<td>0.20</td>
<td>800</td>
<td>85.0</td>
<td>15.0</td>
</tr>
<tr>
<td>2</td>
<td>0.70</td>
<td>600</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>3</td>
<td>2.80</td>
<td>600</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>4</td>
<td>3.20</td>
<td>800</td>
<td>85.0</td>
<td>15.0</td>
</tr>
<tr>
<td>5</td>
<td>4.00</td>
<td>800</td>
<td>85.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

RAM-D5 and HCTZ-$^{13}$C, D2 which are both commercially available and their retention behaviors are similar to those of the target analytes, were used as internal standards. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed.

During method development we observed that a reconstitution solution of water: methanol (80:20%, v/v) + 1% acetic acid gives high peak area and good resolution for both RAM and HCTZ, so it was considered to be used as a reconstitution solution.

Optimization of tandem mass spectrometric conditions

The MS optimization was performed by direct infusion of solutions of both HCTZ and RAM into the electrospray ionization source of the mass spectrometer and to provide optimum sensitivity and selectivity the ESI source operated in the positive ion mode for RAM and in negative ion mode for HCTZ.

The parent ions observed in the product ion mass spectrum were at m/z 417.1 for RAM (Fig.2), m/z 422.1 for RAM-D5 (IS) (Fig.3), m/z 295.9 for HCTZ (Fig.4) and m/z 298.9 for HCTZ-$^{13}$C, D2 (IS) (Fig.5).

Figure 2: Infusion product ion mass spectrum of RAM (parent ion m/z = 417.1)

Figure 3: Infusion product ion mass spectrum of RAM-D5 (parent ion m/z = 422.1)
Method validation

The linearity of the method was evaluated by preparing six calibration curves of eight concentration points of both HCTZ and RAM. The peak-area ratios of the drug to the IS versus the concentrations of drug were plotted to determine the slope, intercept and correlation coefficient ($r^2$) by suitable linear regression weighted (1/x) to demonstrate the linearity of the method. Good linearity was observed over the concentration ranges of 1-150 ng/ml of HCTZ and 0.2-20 ng/ml of RAM with correlation coefficients of 0.9999 and 0.9968 for HCTZ and RAM respectively as shown in Table 2. The calibration curve would be accepted on the basis of the accuracy of ± 20% for LLOQ concentration and ± 15% for the other concentrations [11, 12].

Table 2: Extrapolated linear regression equation and LLOQ of RAM and HCTZ, with dynamic range from replicate calibration curves

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LLOQ (ng/mL)</th>
<th>Correlation coefficient</th>
<th>Linear regression equation</th>
<th>Range (ng/mL)</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAM</td>
<td>0.2</td>
<td>0.9968</td>
<td>$y = 0.1551x - 0.0348$</td>
<td>0.2-20.0</td>
<td>RAM</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.9999</td>
<td>$y = 0.0153x + 0.0054$</td>
<td>1.0-150.0</td>
<td>HCTZ</td>
</tr>
</tbody>
</table>

Accuracy and precision

Intra- and inter-day accuracy and precision were determined by running analytical batches containing calibration curve and six replicates from LLOQ and each level of quality control samples in three separate days. The precision of the method was described as relative standard deviation (RSD) among each day. The accuracy was described as a percentage error of measured concentrations versus nominal concentrations. The method showed good accuracy and precision. Table 3 shows a summary of intra- and inter-day accuracy and precision for both HCTZ and RAM.

Table 3: Precision and accuracy of RAM and HCTZ, human plasma QC samples.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked Concentration (ng/mL)</th>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 24)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (%)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured Concentration (ng/mL)</td>
<td>Precision</td>
<td>Accuracy</td>
<td>Measured Concentration (ng/mL)</td>
<td>Precision</td>
<td>Accuracy</td>
<td></td>
</tr>
<tr>
<td>RAM</td>
<td>0.2</td>
<td>0.198 ± 0.008</td>
<td>4.04</td>
<td>99.00</td>
<td>0.198 ± 0.013</td>
<td>6.62</td>
<td>98.94</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.606 ± 0.020</td>
<td>3.30</td>
<td>99.60</td>
<td>0.595 ± 0.021</td>
<td>3.47</td>
<td>99.20</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9.946 ± 0.460</td>
<td>4.62</td>
<td>97.70</td>
<td>9.717 ± 0.529</td>
<td>5.44</td>
<td>97.17</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>15.758 ± 0.803</td>
<td>5.10</td>
<td>96.09</td>
<td>15.449 ± 0.797</td>
<td>5.16</td>
<td>96.56</td>
</tr>
<tr>
<td>HCTZ</td>
<td>1.0</td>
<td>0.866 ± 0.052</td>
<td>6.00</td>
<td>86.60</td>
<td>0.850 ± 0.045</td>
<td>5.32</td>
<td>85.00</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>2.908 ± 0.061</td>
<td>2.10</td>
<td>96.93</td>
<td>2.914 ± 0.073</td>
<td>2.52</td>
<td>97.13</td>
</tr>
<tr>
<td></td>
<td>75.0</td>
<td>74.014 ± 1.465</td>
<td>1.98</td>
<td>98.69</td>
<td>74.280 ± 1.527</td>
<td>2.06</td>
<td>99.04</td>
</tr>
<tr>
<td></td>
<td>120.0</td>
<td>118.028 ± 1.812</td>
<td>1.54</td>
<td>98.36</td>
<td>116.769 ± 2.220</td>
<td>1.90</td>
<td>97.31</td>
</tr>
</tbody>
</table>
Recovery
Absolute recovery was evaluated by comparing the peak areas obtained from extracted spiked plasma standards with peak areas from standards in the reconstituted solution (80:20 % v/v, water:methanol+1 % acetic acid). The mean absolute recoveries of RAM were 93.30, 96.57 and 92.40 % for the concentration levels of 0.6, 10.0 and 16.0 ng/mL and 97.72 for RAM-D5. HCTZ mean absolute recoveries were 91.98, 95.62 and 97.76 % for concentration levels of 3.0, 75.0 and 120.0 ng/mL and 96.62 for HCTZ-13C,D2 as shown in Table 4.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked concentration (ng/mL)</th>
<th>Mean matrix effect %</th>
<th>Mean % recovery (n =6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAM</td>
<td>0.6</td>
<td>79.03</td>
<td>93.30</td>
</tr>
<tr>
<td>RAM-D5</td>
<td>10.0</td>
<td>80.01</td>
<td>96.57</td>
</tr>
<tr>
<td>HCTZ</td>
<td>3.0</td>
<td>101.34</td>
<td>91.98</td>
</tr>
<tr>
<td>HCTZ-13C,D2</td>
<td>1000.0</td>
<td>105.34</td>
<td>96.62</td>
</tr>
</tbody>
</table>

Specificity and matrix effect
Six different human plasma samples from different sources were prepared and reconstituted with 200 μL of the reconstitution solution as blank samples. Another six blank samples (matrix) were reconstituted with 200 μL of the standard solutions of low quality control (LQC), mid quality control (MQC) and high quality control (HQC) of HCTZ and RAM and analyzed to investigate their intensity change compared to the direct injection of six samples of these standard solutions of LQC, MQC and HQC of HCTZ and RAM. It is considered there is no matrix effect if the deviation of the mean test responses were within 15% of freshly prepared or comparison samples. Matrix effect was calculated [13,14] as per the following equation:

Matrix effect = [(analyte peak area of extracted plasma residue/analyte peak area of neat solution) x100]-100.

The biological matrix was affected on RAM with a −20.97, −19.99, −19.01% factor loss from its intensity for LQC, MQC and HQC respectively and −19.6% for IS. The biological matrix was affected on HCTZ with a 7.99, 5.12, 7.14% factor enhancement on its intensity for LQC, MQC and HQC respectively and 5.34% for IS. The obtained data of matrix effect are shown in Table 3.

Stability
The stability of RAM and HCTZ in plasma was studied under a variety of storage and handling conditions using the QC (three replicates for both QC low and QC high). The bench top stability was assessed by analyzing QC samples that were kept at room temperature for 44 h. Freeze–thaw stability of the samples were obtained over three freeze–thaw cycles, by thawing the QCs at room temperature for 1 h and refrozen for 12–24 h. Auto-sampler stability of drugs and internal standards were tested by analysis of processed and reconstituted QCs, which were stored in the auto-sampler tray at (10°C) for 44 h. Also, after the evaporation step in the extraction method, the stability of the dry extract was evaluated under refrigeration conditions (2-8°C) for the same period (44 h). Stabilities of RAM and HCTZ were tested after storage of QCs at approximately -30°C for 150 Days. The results of stability experiments showed that no significant degradation occurred either at room temperature (25°C), or at autosampler temperature(10°C), or the dry extract in refrigerator at (2-8°C), all for 44 hours, and at -30°C for 150 Days so the three freeze–thaw cycles for RAM and HCTZ plasma samples. The determined concentration of drugs in plasma for various kinds of stability experiments are showed in Table 5.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked Concentration (ng/mL)</th>
<th>Autosampler at 10°C for 44 h</th>
<th>Accuracy (%)</th>
<th>Bench top at 25°C for 44 h</th>
<th>Accuracy (%)</th>
<th>Dry extract at (4-8°C) for 44 h</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAM</td>
<td>0.6</td>
<td>0.573 ± 0.036</td>
<td>95.50</td>
<td>0.620 ± 0.022</td>
<td>103.33</td>
<td>0.608 ± 0.058</td>
<td>101.33</td>
</tr>
<tr>
<td>HCTZ</td>
<td>3.0</td>
<td>2.928 ± 0.045</td>
<td>97.60</td>
<td>2.912 ± 0.081</td>
<td>97.07</td>
<td>2.769 ± 0.077</td>
<td>92.30</td>
</tr>
<tr>
<td></td>
<td>120.0</td>
<td>114.927 ± 0.695</td>
<td>95.77</td>
<td>112.564 ± 1.996</td>
<td>93.80</td>
<td>111.160 ± 2.826</td>
<td>92.63</td>
</tr>
</tbody>
</table>
Stock solution stability

The stability of stock solution of drugs and internal standards were evaluated at room temperature for 22 hours and under refrigeration conditions (2-8°C) for 149 days. The stability was tested by comparing the instrument response with that of freshly prepared solutions. Stability tests proved the stability of RAM, HCTZ and their internal standards at room temperature and under refrigeration conditions.

Clinical study application

The validated method has been applied to monitor RAM and HCTZ concentrations in human plasma samples after oral administration of single dose of Tritace® Comp as a reference product. The pharmacokinetic parameters $C_{\text{max}}$, $AUC_{0-t}$ and $AUC_{0-\infty}$ were calculated and presented in Table 6. The parameters were compared with the results of the test product. The study was accomplished in accordance to the EMA bioanalytical method validation guideline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>$AUC_{0-72}$ (ng.h/mL)</th>
<th>$AUC_{0-\infty}$ (ng.h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAM reference product</td>
<td>15.0 ± 5.75</td>
<td>21.7 ± 11.3</td>
<td>21.7 ± 11.4</td>
</tr>
<tr>
<td>RAM test product</td>
<td>16.2 ± 7.77</td>
<td>21.7 ± 12.4</td>
<td>21.7 ± 12.5</td>
</tr>
<tr>
<td>% Ratio(test/reference)</td>
<td>108.015</td>
<td>100.085</td>
<td>100.025</td>
</tr>
<tr>
<td>HCTZ reference product</td>
<td>131 ± 42.6</td>
<td>933 ± 259</td>
<td>939 ± 273</td>
</tr>
<tr>
<td>HCTZ test product</td>
<td>117 ± 33.5</td>
<td>909 ± 230</td>
<td>992 ± 235</td>
</tr>
<tr>
<td>% Ratio(test/reference)</td>
<td>88.860</td>
<td>97.502</td>
<td>103.438</td>
</tr>
</tbody>
</table>

Clinical Raw Data Screening

The mean plasma RAM and HCTZ concentration/time profile of 27 subjects for reference and test products is presented in Figures 6 and 7.

Figure 6: Mean plasma concentration time profile after the administration of RAM (test (B)) and (reference (A)) 5 mg tablet of 27 subjects.
CONCLUSIONS
A rapid, sensitive and selective method for the determination of RAM and HCTZ in human plasma was developed at LLOQ of 0.2 and 1.0 ng/mL for RAM and HCTZ respectively using liquid chromatography with tandem mass spectrometric detection. This method has been applied to a bioequivalence study in which 27 healthy volunteers were each given a 5/25 mg RAM/HCTZ tablet (reference and test) as a single oral dose.

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