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# Development of a simple and rapid method for the determination of cimetidine in human plasma by high performance liquid chromatography-mass spectrometry (HPLC-MS/MS): Application to a bioequivalence study

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**Bioanalytical methods for bioequivalence studies require high sensibility and rapidity due to the large number of samples and the low plasma concentration of drugs. The present study aimed to develop and validate a high-performance liquid chromatography coupled to sequential mass spectrometry (HPLC-MS/MS) method to quantify cimetidine (CMT) in human plasma and to apply it in a bioequivalence study. CMT and the internal standard, ranitidine, were extracted from plasma by liquid-liquid extraction. After extraction, the samples were analysed by HPLC-MS/MS. The chromatographic separation was performed with a C18 column, and the mobile phase was composed of acetonitrile and ammonium acetate buffer 10 mM to which 5% isopropyl alcohol and 0.1% formic acid were added. The recovery of CMT was 67.14% in a linear range from 25 to 6000 ng ml$^{-1}$. The intraday and interday precision and accuracy were within specified limits. In conclusion, the developed method was precise and accurate and was successfully applied to the bioequivalence study of two formulations of CMT.**

**Key words:** Cimetidine, bioequivalence, HPLC-MS/MS, validation.

## INTRODUCTION

Cimetidine (CMT) (*N*-cyano-*N*-methyl-N[2-[(5-methyl-1H-imidazol-4yl)methyl]thio]ethyl]-guanidine) (Figure 1) is a potent competitive antagonist of histamine at $H_2$ receptors. CMT selectively inhibits gastric acid secretion and reduces the production of pepsin. It is used to treat gastric and duodenal ulcers (Ashiru et al., 2007; Iqbal et al., 2004; Shamsipur et al., 2002; Zendelovska and Stafilov, 2003). Chemically, this imidazoline compound is a white or almost white crystalline powder. It is soluble in alcohol and polyethylene glycol 400, very slightly soluble in

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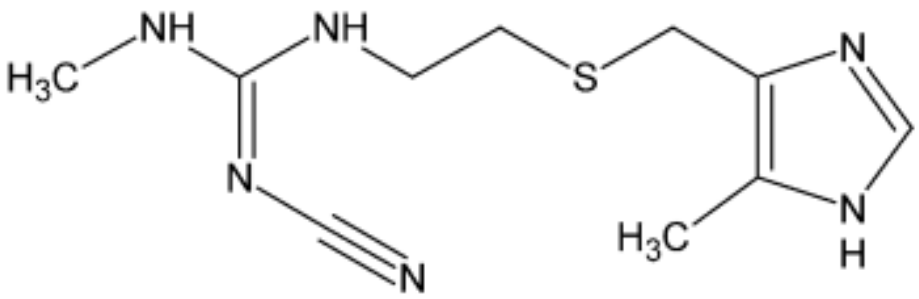

**Figure 1.** Cimetidine (CMT).

chloroform, and insoluble in ether. It has a molecular weight of 252.34 mg $ml^{-1}$ (USP 30 - NF25). According to the Biopharmaceutical Classification System (BCS), CMT is a Class III substance, with low permeability and high solubility (Kawabata et al., 2011). CMT is rapidly absorbed after oral administration. The peak maximum plasma concentration ($C_{max}$) occurs 1 to 2 h after oral administration and it has an elimination half-life of 2 to 3 h. The bioavailability of cimetidine ranges from 62 to 100% in healthy volunteers. Its major route of excretion is through the kidney. Approximately 70% of the drug is excreted in its unchanged form (Jantratid et al., 2006; Miyazaki et al., 2001; Webster et al., 1981).

Several analytical methods for the determination of CMT in human plasma have been published, including high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Abdel-Rahim et al., 1985; Ashiru et al., 2007; Chiou et al., 1989; Hempenius et al., 1998; Iqbal et al., 2004; Jantratid et al., 2007; Kelly et al., 1995; Larsen et al., 1979; Russel et al., 1994; Strong and Spino, 1987; Zendelovska and Stafilov, 2003), and capillary electrophoresis with ultraviolet detection (CE-UV) (Lukša and Josić, 1995). However, the HPLC-UV and CE-UV methods have some limitations. Sample preparation is time consuming and requires a large volume of plasma (over 250 µl). In addition, the limits of quantification are relatively high.

Other quantification techniques for CTM in human plasma have also been used. Jenko et al. (1983) reported the qualitative characterization of CMT and its degradation products based on a combination of HPLC, high performance thin layer chromatography (HPTLC), and fast atom bombardment mass spectrometry (FAB-MS). Xu et al. (1999) developed and validated a quantification method for CMT in plasma using liquid chromatography coupled to mass spectrometry with atmospheric pressure chemical ionization (LC/MS/APCI). The main objective of the present study was to develop and validate a bioanalytical method using liquid chromatography coupled to sequential mass spectrometry (LC-MS/MS) to quantify CMT in human plasma while adhering to good laboratory practice requirements. This simple, practical and rapid method has been applied in a bioequivalence study for two CMT formulations.

## MATERIALS AND METHODS

### Standards

The cimetidine Brazilian Pharmacopoeia (100.2%) and Ranitidine Chloride Brazilian Pharmacopoeia (100.2%) were used as the reference standard and internal standard (I.S.), respectively. Both are chemical reference compounds of the Brazilian Pharmacopoeia and were acquired from the Instituto Nacional de Controle de Qualidade em Saúde (INCQS, Rio de Janeiro, Brazil).

### Reagents

All solvents used were HPLC grade. The ammonium acetate was purchased from Merck (E. Merck, Darmstadt, Germany) and the ultrapure water was obtained from Milli-Q equipment (Millipore Corporation – Massachusetts, USA). Formic acid (E. Merck, Darmstadt, Germany), acetonitrile (ACN), ethyl acetate (EAC) and isopropanol were obtained from J. T. Baker (Phillipsburg, NJ, USA). The dichloromethane (DCM) and methyl tert-butyl ether (MTBE) was provided by Mallinckrodt Chemicals (Phillipsburg, NJ, USA). The blank plasma that was used for the preparation of the standards and for the quality control was obtained from Hemocenter of Pernambuco, HEMOPE (Recife, Pernambuco, Brazil).

### Equipment

A Shimadzu HPLC (Shimadzu Corporation – Tokyo, Japan) with two pumps (LC 10ADvp), a column oven (CTO 10Avp), an autosampler (SIL 10ADvp), and a system controller (SCL 10Avp) were used. The mass spectrometer was a Quattro-LC (Micromass®, Manchester, United Kingdom), triple quadrupole with electrospray ionization. Control and data processing was with Masslynx v3.5 (Micromass®, Manchester, United Kingdom) software. During the sample extraction process, a Jouan M23i (St. Herblaim, France) refrigerated centrifuge was used. The plasma samples were stored at -70°C in a REVCO freezer (Asheville, NC, US) until analysis.

### Chromatographic and spectrometric conditions

The mobile phase was a mixture of acetonitrile and ammonium acetate buffer (10 mmol $ml^{-1}$, adjusted to pH 6.4 with ammonium hydroxide solution) (85:15, v/v) with the addition of 5% isopropanol and 0.1% formic acid pumped with an isocratic flow of 1.5 ml $min^{-1}$.

Separation was performed using a Phenomenex® Gemini $C_{18}$ column (5 µm, 150 × 4.6 mm) (Phenomenex Inc., Torrance, California, USA) and a pre-column Phenomenex® $C_{18}$, wrapped in a column oven at 40°C. The injection volume of each sample was 30 µl. The HPLC eluent was split 1:10 to 150 µl $min^{-1}$ into the mass spectrometer. The mass spectrometer was operated using an electrospray source configured to positive ion mode (ESI+) and acquisition was done using multiple-reaction-monitoring (MRM). The mass transitions monitored were 253.16 > 159.04 and 315.15 > 176, for CMT and I.S., respectively. The spectrometric conditions were: capillary voltage of 3 kV, cone voltage of 18 V, source temperature of 100°C, and collision energy ($E_{col}$) maintained at 15 eV in the presence of argon gas (Ar) at a pressure of $1.88 \times 10^{-3}$. The MRM data were determined with MassLynx (Micromass) software version 3.5.

#### Stock solution, internal standard solution, and plasma standard and quality control samples

The stock solution of CMT (Solution A) for preparation of standard plasma samples was made by dissolving 10 mg of CMT in 10 ml of acetonitrile:water (1:1) (concentration = 1000 µg $ml^{-1}$). The standard plasma samples at concentrations of 25, 50, 100, 300, 600, 1000, 2000, 4000 and 6000 ng $ml^{-1}$ were obtained by successive dilutions of solution A into blank plasma. The plasma samples for quality control (QC) at concentrations of 75, 1500 and 5000 ng $ml^{-1}$ were made by successive dilutions of Solution B (prepared in the same manner as Solution A). The internal standard solution was made by dissolving 10 mg of RNT in 10 ml of acetonitrile: water (concentration = 1000 µg $ml^{-1}$) (Solution C). By diluting Solution C in acetonitrile: water (1:1), a RNT 10 µg $ml^{-1}$ solution was obtained.

#### Preparation of plasma samples for quantification

The extraction was carried by adding 100 µl of plasma and 100 µl of sodium hydroxide 2 mol to tubes containing 50 µl of internal standard solution (RNT 10 µg $ml^{-1}$) in acetonitrile: water (1:1) and homogenizing by vortex mixing for 10 s. Then, 1.5 ml of dichloromethane:ethyl acetate (1:1) was added and the mixture was vigorously shaken for 60 s. After centrifugation (10000 rpm, 5 min, 4°C), the organic phase was transferred to a test tube and dried under nitrogen flow at 40°C. The residue was reconstituted in 500 µl of acetonitrile: water (1:1) for analysis.

#### Validation

Validation tests were performed by determining selectivity, linearity, recovery, limit of quantification, precision, accuracy, and stability. The mean value of accuracy should be within 15% of the actual value except at the low limit of quantification (LLQ), where it should not deviate by more than 20%. The precision determined at each concentration should not exceed 15% of the standard deviation (SD) except for the LLQ, where it should not exceed 20% of SD. All parameters were defined according to resolution 899/03 of the Agência Nacional de Vigilância Sanitária (ANVISA) and The Food and Drug Administration (USFDA) guidelines, which provide a guide for validation of analytical and bioanalytical methods.

#### Bioavailability study

The analytical method developed and validated as was applied to a bioequivalence study with an open-label, cross-over 2 × 2, two-period design. There was a seven day washout interval between doses. A single dose of two tablets of CMT 200 mg (equivalent to 400 mg of CMT) taken with 200 ml of water was administrated to 26 healthy volunteers who fasted for eight hours before and two hours after drug administration. Blood samples were collected into a heparinized tube at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10, 12 and 14 h after administration. The blood samples were centrifuged at 2400 rpm for 5 minutes and the plasma removed and stored frozen at -70° C until analysis.

Plasma samples from volunteers were analyzed interspersed the calibration curve formed by plasma samples with the standard in nine concentrations (25, 50, 100, 300, 600, 1000, 2000, 4000 and 6000 ng·$mL^{-1}$) and the quality control plasma samples in three different concentrations (low: 75 ng$mL^{-1}$; medium: 1500 ng·$mL^{-1}$ and high: 5000 ng·$mL^{-1}$).The clinical protocol was approved by the Ethics Committee of Universidade Federal de Pernambuco (SISNEP FR-106744). The study was carried out according to the declaration of Helsinki (1965) and Tokyo (1975), Venice (1983), South Africa (1996) and ANVISA's 196/96 and 251/97 resolutions.

## RESULTS AND DISCUSSION

### Validation

Figure 2 shows the chromatogram obtained under the established conditions. The retention times for CMT and RNT were 1.39 and 1.46 min, respectively. Note that the total run time is 2.5 min, which is much less than that of other methods (Hempeniu et al., 1998; Sun et al., 2009; Zendelovska and Stafilov, 2003). This makes the present approach more suitable for application in bioavailability studies and reduces solvent waste. The selectivity of the method can be attested, no interfering peaks were observed with the same retention time of analyte and IS, when mass spectrometry detection was used for the analysis of plasma samples from different volunteers, including lipemic and hemolysed ones. Figure 2 show the chromatogram blank of plasma with an absence of sample interfering at regions where analytes, CMT and RNT, are eluting.

### Linearity and limit of quantification

Linearity was established by analysis of standard plasma samples in triplicate. The correlation coefficient was 0.994. (Table 1). The LLQ was 25 ng $ml^{-1}$. The LLQ was defined as the smallest amount of analytes in samples with an acceptable accuracy, that is, a coefficient of variation of 6 to 10%. The method provided excellent detectability, with a suitable LLQ value for carrying out the tests that is lower than most previously published methods (Hempeniu et al., 1998; Jantratid et al., 2007; Zendelovska and Stafilov, 2003).

### Recovery

The liquid-liquid extraction (LLE) with the highest recovery was the mixture of dichloromethane: ethyl acetate (1:1 v/v), at a ratio of 1 part of plasma to 10 parts of organic mixture, in alkaline media. These values were

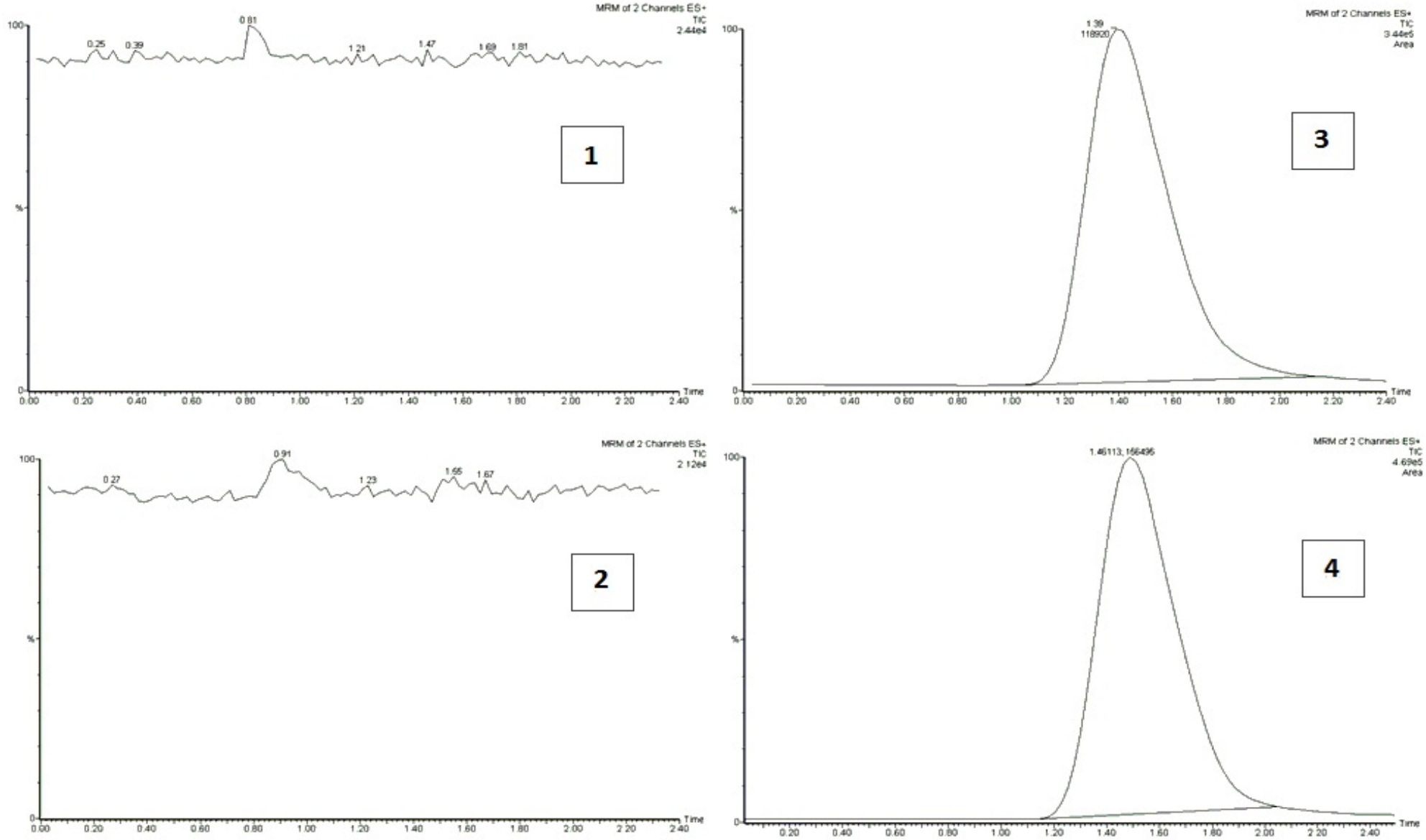


**Figure 2.** Chromatograms illustrating the analysis of normal plasma pool (1), hemolysed.

**Table 1.** Precision and accuracy of concentration levels of linearity test.

| Nominal value (ng ml$^{-1}$) | Observed value (ng ml$^{-1}$) (Mean ± SD) (n=13) | Precision (%) | Accuracy (%) |
|---|---|---|---|
| 25 | 23.96 ± 1.46 | 6.10 | 95.9 |
| 50 | 54.31 ± 1.46 | 2.68 | 108.6 |
| 100 | 97.34 ± 3.56 | 3.66 | 97.3 |
| 300 | 322.61 ± 1.91 | 0.59 | 107.5 |
| 600 | 604.78 ± 27.33 | 4.52 | 99.2 |
| 1000 | 978.04 ± 50.67 | 5.18 | 97.8 |
| 2000 | 1838.36 ± 101.25 | 5.51 | 91.9 |
| 4000 | 3880.59 ± 265.86 | 6.85 | 97.0 |
| 6000 | 6186.70 ± 370.37 | 5.99 | 103.1 |

obtained by the ratio between the areas of the chromatograms in plasma and in solution, at their respective levels (75, 1500 and 5000 ng ml$^{-1}$). By optimizing the process, we obtained a recovery of 67.14 and 90.02% for CMT and RNT, respectively (Table 2). The recovery of the drugs was enhanced in alkaline media, that is by adding 100 µl of NaOH at 2 mol ml$^{-1}$. This can be explained by the fact that both CMT and RNT are weak bases and are unionized in alkaline pH (Jantratid et al., 2006). Increasing the ratio of solvent led to a considerable increase in recovery for both drugs. This increase is partly due to increasing the amount of solvent available so that saturation at the organic portion (solvent) does not occur. The increase in recovery is also caused by a reduction of the plasma matrix effect, decreasing the ion suppression effect caused by co-extraction of matrix components (Ardrey, 2003; Ribani et al., 2004). The extraction method used here requires only 100 µL of plasma for analysis, which is a significant advantage over other methods (Hempenius et al., 1998; Iqbal et al., 2004; Kunitani et al, 1981; Strong and Spino, 1987; Zendelovska and Stafilov, 2003). Furthermore, the LLE applied in this study proved to be simple, fast and inexpensive.

**Table 2.** Results of CMT and RNT (IS) recovery.

| Concentration (ng/ml) | Recovery standard (%) | SD (%) |
|---|---|---|
| 75 | 67.05 | 5.38 |
| 1500 | 65.16 | 3.20 |
| 5000 | 68.22 | 7.39 |
| Mean | 67.14 | - |
| RNT | 90.02 | 5.32 |

SD – standard deviation, CMT – cimetidine, RNT – ranitidine.

## Precision and accuracy

For precision and accuracy studies, 6 replicates of each concentration of QC sample were prepared and analysed the same day (intra-day precision and accuracy) or prepared and analysed on 3 consecutive days (inter-day precision and accuracy). The intraday and interday precision and accuracy of the method for each QC concentration and for the LLQ are presented in Table 3. The inter-day precision and accuracy of the CMT assays was within 15% whilst the intra-day precision and accuracy was within 20% of the nominal concentration. These results were within the limits of the measurement. The precision and accuracy of the method were also evaluated during its application to quantify collecting points for the 26 volunteers, through 52 quality controls at each level, which were interspersed with analysis. The results, displayed in Table 4, highlight the excellent precision and accuracy obtained during the application of the method, where 884 samples from volunteers were analysed.

## Stability study

Plasma samples for QC were prepared before the analysis began. Blank plasma samples (free drug) and plasma samples for QC that received CMT remained stable during freezing and melting tests, 36 h post-processing, short term 6 h on the bench, and after 30 days in a long-term study at -20 and -70°C (Table 4). The samples in solution also remained stable for 48 h at room temperature and 72 h at 4°C. Table 5 shows the data obtained from the reference samples and from the three freezing and melting cycles at the specified temperatures (-20 and -70°C). It also shows the coefficient of variation for the samples after the third freezing and melting cycle at different temperatures. The study showed, with 95% confidence, that there is no statistically significant difference among storage temperatures for the three freezing and melting cycles. The same can be said for the long term stability (Table 6). Therefore, samples of CMT in human plasma can be stored at a temperature between -20 and -70°C.

**Table 3.** Inter and Intra-day precision and accuracy of cimetidine.

| Nominal value | Intra-day (ng ml$^{-1}$) (n=6) | | | Inter-day (ng ml$^{-1}$) (n=24) | | |
|---|---|---|---|---|---|---|
| (ng ml$^{-1}$) | Mean ± SD | Prec. (%) | Acc. (%) | Mean ± SD | Prec. | Acc. |
| 25 | 25.99 ± 3.12 | 12.02 | 103.96 | 26.05±3.18 | 12.20 | 104.2 |
| 75 | 74.21 ± 2.06 | 2.78 | 98.94 | 74.46± 5.69 | 7.65 | 99.28 |
| 1500 | 1387.07 ± 43.96 | 3.17 | 92.47 | 1387.07± 67.48 | 4.86 | 92.47 |
| 5000 | 4990.61± 83.7 | 1.68 | 99.81 | 4988.27± 322.7 | 6.47 | 99.77 |

SD – standard deviation, Prec. – precision, Acc. – accuracy.

**Table 4.** Precision and accuracy results' with 26 volunteers.

| Level | LQC (N =52) | MQC (N = 52) | HQC (N = 52) |
|---|---|---|---|
| Mean (µg/ml) | 73.47 | 1433.11 | 4814.24 |
| SD | 7.40 | 110.37 | 315.80 |
| Precision (%) | 10.07 | 7.70 | 6.56 |
| Accuracy (%) | 97.96 | 95.54 | 96.28 |

LQC – low quality control, MQC – middle quality control, HQC – high quality control, SD – standard deviation.

**Table 5.** Data from stability study of CMT.

| Parameter | Cimetidine (n=4) | | | |
|---|---|---|---|---|
| Stability | Nominal conc. (ng ml$^{-1}$) | Mean concentration (±SD) (ng ml$^{-1}$) | CV (%) | Accuracy (%) |
| Short-term[A] | 75 | 76.803 (±7.282) | 9.48 | 103.63 |
| | 5000 | 4719.849 (±36.221) | 0.77 | 95.95 |
| Post-processing[B] | 75 | 72.649 (±0.756) | 1.04 | 99.07 |
| | 5000 | 4846.987 (±254.919) | 5.26 | 96.95 |
| Freezing and melting[C] | 75 | 71.851 (±3.278) | 4.56 | 94.25 |
| | 5000 | 4869.926 (±319.908) | 6.57 | 94.73 |
| Freezing and melting[D] | 75 | 73.222 (±4.015) | 5.48 | 96.05 |
| | 5000 | 4652.545 (±240.962) | 5.18 | 100.57 |
| Long-term [E] | 75 | 71.727 (±7.401) | 10.32 | 97.08 |
| | 5000 | 4537.545 (±182.003) | 4.01 | 102.25 |
| Long-term [F] | 75 | 76.028 (±8.626) | 11.35 | 102.86 |
| | 5000 | 4890.518 (±298.490) | 6.10 | 110.20 |
| Sample in solution[G] | 75 | 75.813 (±6.282) | 5.42 | 101.23 |
| | 5000 | 4989.849 (±39.234) | 0.79 | 99.94 |
| Sample in solution[H] | 75 | 75.354 (±1.614) | 2.14 | 100.47 |
| | 5000 | 4885.49 (±157.94) | 3.23 | 97.7 |

[A]At room temperature (23°C) for 6 h, [B]auto-injector at 4°C for 36 h, [C]after three cycles at -20°C, [D]after three cycles at -70°C, [E]long at -20°C (30 days), [F]long at -70°C (30 days), [g]at room temperature (23°C) for 48 h, [H]at 4°C for 72 h.

**Table 6.** Analysis of variance (ANOVA) from the stability of cimetidine.

| Study | Temperature (°C) | Level (ng ml$^{-1}$) | DF | F calculated | F critical |
|---|---|---|---|---|---|
| Freezing and melting* | -20 | 75 | 11 | 0.28711 | |
| | -70 | 5000 | 11 | 0.74549 | |
| | | | | | 4.25649 |
| Long-term** | -20 | 75 | 11 | 0.26112 | |
| | -70 | 5000 | 11 | 3.40332 | |

*3 cycles of freezing and melting, **30 days, DF = Degrees of freedom

**Table 7.** Pharmacokinetics parameters of the test and reference formulations.

| Pharmacokinetics parameter | Test formulation (mean ± SD) | Reference formulation (mean ± SD) |
|---|---|---|
| $AUC_{(0-t)}$ (ng/ml.h) | 3192.23 ± 721.86 | 3151.14 ± 629.28 |
| $AUC_{(0-inf)}$ (ng/ml.h) | 3329.87 ± 730.52 | 3281.31 ± 634.75 |
| $C_{max}$ (ng/ml) | 850.73 ± 194.69 | 867.78 ± 243.78 |
| $T_{max}$ | 1.82 ± 0.97 | 1.70 ± 1.14 |
| T ½ | 2.61 ± 0.60 | 2.69 ± 0.70 |
| $K_{el}$ (h/h) | 0.28 ± 0.06 | 0.27± 0.07 |

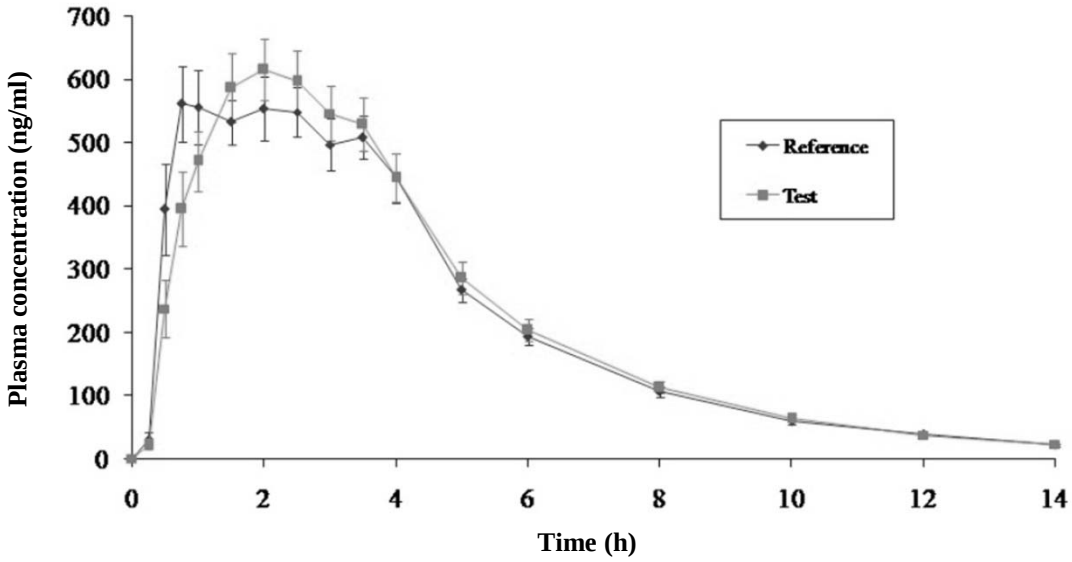


**Figure 3.** "Plasma concentration *versus* time" curve.

## Bioequivalence study

Figure 3 shows two mean "plasma concentration versus time" curves obtained after administration of reference formulation CMT 400 mg (Tagamet®, 200 mg tablets) and a test formulation. Table 7 shows the pharmacokinetics parameters for both formulations. The confidence interval (90%) for $C_{max}$ was 88.04% (lower limit) and 110.23% (upper limit), obtained through statistical analysis with the shortest tool. The $C_{max}$ geometric mean, calculated from logarithmically transformed data was 838.670 for the reference formulation and 826.408 for the test formulation, resulting in a ratio ($C_{max}$ test/$C_{max}$ reference) of 98.54 with a test power of 94.83%. The area under the concentration-time curve from time zero to the last collection time, $AUC_{(0-t)}$ had lower and upper 90% confidence limits of 93.99 and 107.76, respectively. The geometric mean $AUC_{(0-t)}$, calculated from logarithmically transformed data

for the reference formulation was 3090.065 and 3109.814 for the test formulation, resulting in a ratio (test/reference) of 100.64, with a test power for $AUC_{(0-t)}$ of 99.96%. For the area under concentration-time curve from zero to infinity, $AUC_{(0-inf)}$, the lower and upper limits of confidence (90%), calculated by shortest, were 94.50 and 107.68, respectively. The mean $AUC_{(0-inf)}$, calculated for the reference and test formulations from logarithmically transformed data were 3221.131 and 3249.221, resulting in a ratio (test formulation/reference formulation) of 100.87 with a test power of 99.98%.

## Conclusion

The bioanalytical method developed and validated for CMT quantification in plasma samples by LC-MS/MS showed appropriate specificity, sensibility, linearity, robustness, precision and accuracy, allowing for its use in bioequivalence tests and pharmacokinetic studies of CMT. This method has two major advantages: the short time required and the low sample (plasma) volume (only 100 µl) required.

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