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Research Article

# Development of a Bio analytical Assay for the Determination of Rupatadine in Human Plasma and its Clinical Applications

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## Abstract

**Background:** Rupatadine is an antihistaminic drug that is used for the treatment of allergic rhinitis, chronic idiopathic urticarial, additionally, it can be used as safe and effective alternative to loratadine.

**Aim:** Development of bio-analytical method for rapid quantification of rupatadine in human plasma and its clinical application in bioequivalence study of generic and reference products of rupatadine 10mg film coated tablet.

**Methods:** Extracted rupatadine was chromatographed with mobile phase of methanol: 0.5% formic acid 80:20 v/v at flow rate 0.5ml/min, ESI positive mode, and m/z 416→282.1, 383→337 for rupatadine and loratadine as internal standard respectively. The bioequivalence study was conducted in a crossover design invovlving 24 volunteers and pharmacokinetic parameters $AUC_{0-t}$, $AUC_{0-inf,}$ $C_{max}$, and $T_{max}$ were used for assessment of bioequivalence of the two products

**Results:** The average recovery of rupatadine from human plasma was 87.567%, limit of quantitation was 0.01ng/ml, and the correlation coefficient ($r^2$) obtained was 0.9997. Statistical analysis for the pharmacokinetic parameters using ANOVA test showed a non- significant difference between generic and reference products included in the study.

**Conclusion:** The developed bioanalytical LC/MS/MS method is simple, sensitive, precise, accurate and valid for rupatadine quantification in human plasma and is suitable for application in pharmacokinetic and bioavailability studies and therapeutic monitoring of rupatadine in management of allergic disease to ensure effective therapeutic drug levels and avoid potential undesired adverse events. Results of bioavailability study showed that both generic and reference products are bioequivalent and both products can be considered interchangeable in medical practice.

**Keywords:** Rupatadine; Bioanalytical Method Validation; LC/MS/MS; Non-Sedating Antihistamine; Allergic Rhinitis

## Introduction

Rupatadine is a second generation non-sedating antihistamine [1] with platelet-activating factor (PAF) antagonist activity that is used for the treatment of allergic rhinitis and chronic idiopathic urticaria [2].

In a randomized parallel group study comparing the efficacy of rupatadine versus loratadine in the treatment of seasonal allergic rhinitis, rupatadine 10 and 20 mg a day were both clinically effective, with the higher dosage causing a greater improvement in the majority of symptoms. Results showed a clear difference between rupatadine and loratadine treatment groups regarding improvement of symptoms, moreover, the benefits of both rupatadine doses were mainly detected in the significant reduction of sneezing and nasal itching scores in comparison with loratadine 10 mg [3].

Rupatadine which is a platelet-activating factor receptor inhibitor might modulate dengue-associated vascular leak and its effect were assessed in vitro by a dengue model which showed that rupatadine signifcantly reduced endothelial permeability by dengue sera and significantly inhibits the increased haematocrit in dengue-infected mice with dose-dependency [4].

Candidate drug in this study, rupatadine, is marketed as fumarate under brand name Rupafin® 10 mg film coated tablets [5], indicated for symptomatic treatment of allergic rhinitis, urticaria in adults and adolescents over 12 years of age [6] with recommended dosing is 10 mg once per day as a single dose [1].

After single dose administration of rupatadine 20mg tablet, the mean $C_{max}$, $AUC_{o\text{-}inf}$, $T_{1/2}$ was 4.57ng/ml, 18.22ng.hr/ml, and 4.11hr respectively, median $T_{max}$ was 0.75 hour [7]. In another pharmacological profile article the mean $C_{max}$, and $T_{1/2}$ was found to be 2.2ng/ml and 6hr (range 4.3-14.3h) respectively, median $T_{max}$ was 0.75 to 1 hr [8]. Moreover, in an efficacy and safety study of rupatadine reported mean $C_{max}$, $AUC_{0\text{-}24}$, $T_{1/2}$ and $T_{max}$ to be 2.3ng/ml, 7.6ng.hr/ml, 4.6hr, and 0.8hr respectively [9].

On the other hand, the following mean values were obtained after single- or multiple-dose administration of rupatadine 10mg or 20mg for $C_{max}$, $t_{max}$, $AUC_{0\text{-}24}$, $AUC_{0\text{-}\infty}$, and $T_{max}$ 1.9ng/ml, 1.0 hour, 8.4ng.h/ml, 9.2ng.h/ml, and 0.8–1.0 hour independently of the administered dose [10]. The mean values of pharmacokinetic parameters obtained in another study of rupatadine 10mg for $C_{max}$, $AUC_{0\text{-}t}$, $AUC_{o\text{-}inf}$, $T_{1/2}$, and $T_{max}$ were; 4.62 ± 1.51ng/ml, 14.81 ± 5.79ng.hr/ml, 15.39 ± 6.45ng.hr/ml, and 4.76 ± 2.07hr, and 0.67 hr (range 0.67–2.0 hrs)respectively [11].

Different analytical methods are investigated and developed for the evaluation of rupatadine in pharmaceutical dosage form, including, reversed-phase liquid chromatography with UV detector [12], micellar electrokinetic capillary chromatography (MEKC) [13], high performance thin-layer chromatography (HPTLC) [14], methods. Moreover, sensitive isocratic LC-MS/MS methods with positive electrospray ionization (ESI) were developed and validated for the quantitation of rupatadine in plasma [15,16].

Bioanalytical different methods used for the determination of rupatadine in biological samples using LC/MS/MS method showed a lower quantitation limits LLOQ of 0.1ng/ml [17]. and 0.2ng/ml [7].

A sensitive analytical method for determination of rupatadine in biological samples using LC/MS/MS in human plasma was chromatographed on a reversed phase column with a mobile phase of 0.2 M ammonium acetate at pH 4.5 and methanol at flow 1ml/min and gradient elution, mass parameters were set on multiple reaction monitoring mode (MRM) and positive ESI with a quantitation limit (LLOQ) of 0.1 ng/ml and a linear dynamic range of 0.1 ng/ml to 10ng/ml [18].

For a more sensitive and linear dynamic range an LC/MS/MS assay developed in which drug extracted by ethyl acetate from alkalinized human plasma with saturated sodium bicarbonate. Chromatographic separations were achieved on a hedera ODS-2 column using mobile phase of methanol and 10mM ammonium acetate containing 0.1% formic acid in gradient elution program. The method was fully validated. The multiple reaction monitoring was based on m/z 416.0→309.1 for rupatadine, m/z 286.1→217.1 for letrozole (Internal standard), the lower limit of quantitation LLOQ was 0.05 ng/ml, and assay was linear over a concentration range from 0.05 to 35 ng/ml [19].

In this study, a comparative bioavailability study of generic versus reference product of rupatadine 10mg film coated tablet on 24 healthy subjects as per protocol was conducted in which the developed bioanalytical method was used for the determination of rupatadine in human plasma, the design used is an open label randomized crossover design with a washout period of one week between treatments [20]. Analysis of plasma would be done through developing and validating an LC/MS/MS method in compliance with the international guidelines [21]. Pharmacokinetic calculations were performed by WinNonlin program and statistical analysis (ANOVA) were done using SAS software. Sequence effect was tested and the 90% confidence intervals for $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$, and $C_{max}$ were calculated for the ratio or difference between treatments and results showed to be in the limit of 80% to 125% confidence limits [22].

## Materials and Methods

- **Chemicals and Reagents:** Purified water of LC/MS/MS grade, methanol (SIGMA Aldrich, Germany), acetonitrile (Scharlab, Spain), formic acid (Scharlab, Spain), sodium tertaborate (Scharlab, Spain), diethylether (Fisher Scientific, UK), and dichloromethane (Fisher Scientific, UK).
- **Equipment:** Adjustable pippettes (P200, and P1000), disposable plastic pipettes tip yellow (range 5 - 200 µL) and blue (range 200 1000 µL), disposable glass test tubes 120 x 12 mm, Vortex mixer (Boeco, Germany), vacuum pump (Boeco, Germany), PH-meters (Boeco, Germany), water purifier (Purelab option- R7ELGA, U. K.), sonicator (Crest, U.S.A.), analytical balance (Sartorius, U.S.A.), concentrator plus/vacufuge® pus (Eppendorf, Germany), and LC-MS/MS Agilent 6410B Triple Quad, USA.

## Methods

### Analytical method

### Chromatography

In house developed chromatographic conditions was used and mobile phase composition is methanol: 0.5% formic acid 80:20 v/v. The flow rate was set at 0.5ml/min. Injection volume was set at 5ul. MS/MS 6410B detector was operated at ESI positive mode, m/z was 416→282.1, 383→337 for rupatadine and loratadine as internal standard respectively.

Fragmentor energy was set at 135 for rupatadine and loratadine and the collision energy was set at 20 for rupatadine and loratadine.

### Preparation of solutions

### Master standard solution

An accurately weighed 12.8 mg of standard rupatadine fumarate (equivalent to 10mg rupatadine) was transferred into a 100 ml volumetric flask and about 80 ml methanol was added, and sonication was done for 10 minutes, the volume was completed with methanol to obtain a solution containing 100ug/ml rupatadine "Solution A", 0.1ml of it was transferred to 100ml volumetric flask and volume completed with methanol to obtain final concentration of 100 ng/ml of rupatadine "Solution B".

### Working solutions

| Master Solution used | Mililitres taken | Final concentration obtained (ng/ml) | Final volume (ml) |
|---|---|---|---|
| "Solution B " | 0.01ml | 0.1 | 10 |
| "Solution B " | 0.05ml | 0.5 | 10 |
| "Solution B " | 0.1ml | 1 | 10 |
| "Solution B " | 0.5ml | 5 | 10 |
| "Solution B " | 1ml | 10 | 10 |
| "Solution B " | 2ml | 20 | 10 |
| "Solution B " | 3ml | 30 | 10 |
| "Solution B " | 4ml | 40 | 10 |
| "Solution B" | 6ml | 60 | 10 |

**Table a**

### All dilutions are done with methanol

### Loratadine standard solution

Accurately weighed 10mg of standard loratadine was transferred to a 100 ml volumetric flask and about 80 ml methanol was added, and sonication was done for 10 minutes. The volume was completed with methanol to obtain a solution containing 100ug/ml loratadine solution (A). From solution (A) 0.05ml were transferred to a 100ml volumetric flask and volume completed with methanol to obtain 50 ng/ml loratadine solution (B).

### Preparation of standard rupatadine different concentrations in human plasma

The standard samples in human plasma were prepared by transferring a 50 ul aliquot of rupatadine working standard solutions ranging from 0.1 to 60 ng/ml to a centrifuge tubes containing 500 ul of blank human plasma.

### Sample preparation

Plasma samples (500 ul) were transferred into appropriate centrifuge test tubes and 50 ul of loratadine working solution (50ng/ml) was added, then vortex-mix for 1 minute, and 50 ul of 0.25M NaOH was added and vortex-mix for 1 minute, 3ml of diethyl ether: dichloromethane (70:30) v/v was added and vortex-mix was done for 1 to 2 minutes, centrifugation of samples at 4000rpm for 5 minutes and the clear supernatant layer was transferred to clean test tube and evaporated till dryness, finally, the residue was reconstituted with 200ul mobile phase and transferred to vial insert for injection and quantitation on LC/MS/MS.

### Quantitation

The unknown volunteer sample concentration was calculated as per formula: $y = ax + b$, where:

Y: is the response ratio. X: unknown concentration of drug in plasma. a: slope of calibration curve b: Y-Intercept.

### Bioequivalence study

### Study ethics

This study was conducted in accordance with the international conference of harmonization (ICH) and good clinical practice (GCP) guidelines adopted by the european agency for the evaluation of medicinal products (EMEA), and after ethics committee approval on the bioequivalence study protocol of rupatadine 10 mg film coated tablets (study code: HIS-MASP-BES-1115/0224). Essential documents and records were all archived according to drug research center (DRC) internal procedures for authorized direct access.

Written informed consents were reviewed, discussed and signed by the participant and clinical investigator before starting of screening procedure without any obligation on the volunteers to continue if they didn't want to.

Clinical Investigator, study director (principal investigator), licensed physicians responsible for physical examination and following-up of the subjects for appearance of any side or adverse effects, measurement of vital signs throughout the study including blood pressure, pulse rate, body temperature, respiratory rate before and all over the study and registered nurses were responsible for blood sampling.

### Inclusion criteria

Age 18-55 years, Ideal weight within the normal range according to accepted life tables, non-contributory history and normal physiological examination, laboratory data within normal limits, performance and compliance and the subjects should be without known history of alcohol or drug abuse problems and should preferably be non-smokers.

### Exclusion criteria

A known hypersensitivity to the drug, gastrointestinal diseases, auto immune diseases, renal diseases or dysfunction, cardiovascular disease of any type, pancreatic disease including diabetes, hepatic disease, hematological, osteopathic, or pulmonary disease, history of alcoholism or drug abuse, serious psychological illness, positive HIV-I, smoking (if including they should be identified), abnormal (out of range) laboratory values, subject who have taken any medication (Rx or OTC) less than two weeks of the trials starting date, subject who have donated blood or who have been in multiple dosing studies requiring a large volume of blood (more than 500 ml) to be drawn within six weeks preceding the start of the trials.

### Subjects

Twenty-four healthy adult subjects participated in the bioequivalence study were subjected to general physical examination, neurological assessment, urine analysis and blood analysis. The selected subjects had not any history of drug or alcohol abuse. All subjects didn't have any acute or chronic gastrointestinal, cardiac, vascular, hepatic, or renal disease. Concurrent medication was not allowed during the time course of the study, meals, beverages drink, coffee or tea are not allowed for four hours after study dose administration. At 12:00pm the day of dosing they received a standard meal and at about 16:00 pm another stansardized meal was introduced.

### Study design

This study was conducted in a two-way crossover design to compare the bioavailability of rupatadine between generic and reference products in 24 male healthy adults under fasting conditions with a washout period of one week. The number and disposition of the blood collections as well as the wash out period were designed with respect to pharmacokinetic parameters of rupatadine.

### Sample collection

The number of blood collections for drug analysis was 16 samples in each period. The volume of blood taken for the determination of rupatadine in plasma was 5ml per sample at the following sampling intervals: 0 (directly prior to dosing), 10min, 20min, 30min, 45min, 1hr, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, and 48 hours after the administration. Blood sample collection was performed into a tubes containing anticoagulant EDTA disodium and centrifuged at approximately 4000 r.p.m. for 10 minutes. Plasma samples were separated in a 5 ml-plastic wassermann tube and the collected samples were kept at a -80°C freezer until analysis. The total amount of blood withdrawn during the whole study did not exceed 160 ml.

### Analysis of plasma samples

The withdrawn volunteers' samples were analyzed by using LC-MS/MS technique for the quantitation of rupatadine in human plasma.

### Pharmacokinetic calculations

The following pharmacokinetic parameters (variables) of rupatadine were assessed; maximum plasma concentration ($C_{max}$),time point of maximum plasma concentration ($t_{max}$), half-life of drug elimination ($t_{1/2e}$),terminal rate of elimination ($K_e$),area under plasma concentration-time curve from zero to 72 hr ($AUC_{0-72}$).

### Statistical analysis of data

Statistical analysis of the determined pharmacokinetic data was performed using statistical computerized program SAS software for determination of analysis of variance (ANOVA). Bioequivalence conclusion could be demonstrated for rupatadine within the prescribed 90% confidence interval of 80% to 125% for $AUC_{0-t}$, $AUC_{0-inf}$ and $C_{max}$.

## Results

### Analytical validation

- **Chromatograms of Rupatadine:** Rupatadine and its internal standard were well separated with retention time was 0.8, and 1.4 minutes, peaks were sharp, symmetrical with good baseline and minimum tailing, thus facilitating the accurate measurement of the peak response.

- **Linearity, Precision, and Accuracy**: Peak area ratios of different concentration of rupatadine in human plasma over the range of 0.01 to 6 ng/ml was highly linear with correlation coefficient ($r^2$) of 0.9997,the average results of interday variation C.V.% was 0.909%. Accuracy and precision were assessed at within-day and between-day basis at three drug concentrations in the range of expected concentrations, where the results of intra-day and inter-day accuracy showed an average recovery percentage of 99.987% and 99.190% with an average CV% of 0.909%. The results of stability in plasma showed that the average recovery of rupatadine was greater than 95% providing that it is stable in the studied conditions.

### Bioequivalence study

- **Clinical observation:** The drug was well tolerated to all participating subjects, blood sampling from all subjects during the whole study was obtained at the proper time without any recorded incidence of adverse events within the participated subjects.
- **Pharmacokinetic data and assessment of bioequivalence:** The mean values of $C_{max}$, $t_{max}$, $t_{1/2e}$, $AUC_{0-t}$, and $AUC_{0-\infty}$ were 2.652 ± 0.307ng/ml and 2.618 ± 0.287ng/ml, 0.813 ± 0.169hr and 0.802 ± 0.127hr, 5.931 ± 0.174hr and 5.902 ± 0.149hr, 16.525 ± 4.341ng.hr/ml and 16.679 ± 4.195ng.hr/ml, and 16.620 ± 4.345ng.hr/ml and 16.768 ± 4.198 ng.hr/ml for generic and reference products respectively.
- **Statistical Analysis:** The results of 2-way ANOVA of $C_{max}$, $AUC_{0-t,}$ and $AUC_{0-inf,}$ showed that there was no significant difference between generic and reference product. The point estimate (%) results for $C_{max}$, $AUC_{0-t}$, $AUC_{0-inf}$ were 101.241%, 98.631%, and 98.659% respectively. The 90% confidence intervals of parametric means of $C_{max}$, $AUC_{0-t}$, and $AUC_{0-inf}$ were 98.810% to 103.733%, 91.707% to 106.078%, and 91.769% to 106.068% respectively.

## Discussion

The LC/MS/MS method used in this study was simple, of excellent sensitivity, specificity, precision and accuracy. The calibration curve was linear over the concentration range of 0.01 to 6ng/ml and $r^2$ was equal to 0.9997 which is in accordance with FDA Guidelines [21], and so it could be applied for accurate detection and determination of rupatadine in human plasma during clinical, pharmacokinetic and bioavailability studies.

The in house developed chromatographic conditions showed to have LLOQ of 0.01ng/ml which was much lower than those reported in other studies showing LOQs of 0.1ng/ml, 0.2ng/ml, and 0.05ng/ml [7,12-19]. Besides, in this study there was some modifications in chromatographic conditions using isocratic elution method instead of the reported gradient elution methods [18,19].

The use of structurally related internal standard in bioanalytical is an important issue, to ensure accuracy and validity of target drug quantification and to compensate any variation due to sample processing and detector response alterations. Loratadine which is structurally related to rupatadine was used as an internal standard instead of clomipramine and letrozole and others mentioned in literature methods [7,12-19].

In a randomised, double-blind, parallel-group, multicentre clinical trial evaluated the efficacy and safety of rupatadine and cetirizine in the treatment of patients with seasonal allergic rhinitis (SAR). Evaluation of efficacy at the seventh day, 93.3% and 83.7% patients in the rupatadine and cetirizine groups, respectively, showed some or great improvement in symptoms. Runny nose at the seventh day of treatment was absent or mild in 81.1% of patients in the rupatadine group and in 68.6% of patients in the cetirizine group. Adverse events (AEs) were similar in both treatment groups, Somnolence was reported in 9.6% and 8.5% of patients treated with rupatadine or cetirizine, respectively. Suggesting that rupatadine 10 mg may be a valuable and safe alternative for the symptomatic treatment of seasonal allergic rhinitis [23].

As shown previously, the clinical importance of rupatadine in treatment for allergic rhinitis and chronic idiopathic urticarial [2] with better clinical outcomes over loratadine [3] and cetirizine [23]. It is worthy to mention that, it is of high importance to develop a bioanalytical assay in order to ensure accurate and precise therapeutic monitoring and testing the validity of generic drug products for commercial use to ensure achieving the therapeutic goals of rupatadine.

It was found in this work that the obtained mean values of pharmacokinetic results of was in agreement with those reported in the literature which stated that $T_{max}$ were found to be 0.75 hours in average (range from 0.5 to 2 hours), $C_{max}$ ranged from 1.9ng/ml to 4.62 ± 1.51ng/ml, and $T_{1/2}$ were 4.6 hr in average [7-11].

In bioequivalence study 90% confidence interval of 80.00% to 125.00% for $AUC_{0-t}$, $AUC_{0-inf}$ and $C_{max}$ with respect to the parametric method on Ln-transformed data should be fulfilled. In this

study the percentage values of point estimate for $C_{max}$, $AUC_{0-t}$, $AUC_{0-inf}$ were 101.241%, 98.631%, and 98.659% respectively. The 90% confidence intervals of parametric means of $C_{max}$, $AUC_{0-t}$, and $AUC_{0-inf}$ were 98.810% to 103.733%, 91.707% to 106.078%, and 91.769% to 106.068% respectively, thus providing a 90% confidence intervals limits lying within FDA acceptance limits (80% to 125%) [22].

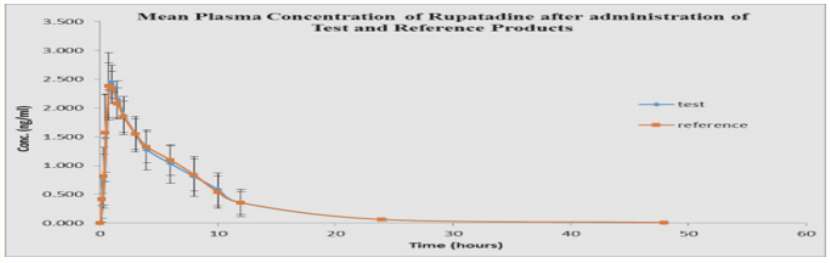


**Figure 1:** Mean Plasma concentration following single dose administration of generic and reference products of rupatadine 10mg Film Coated Tablet.

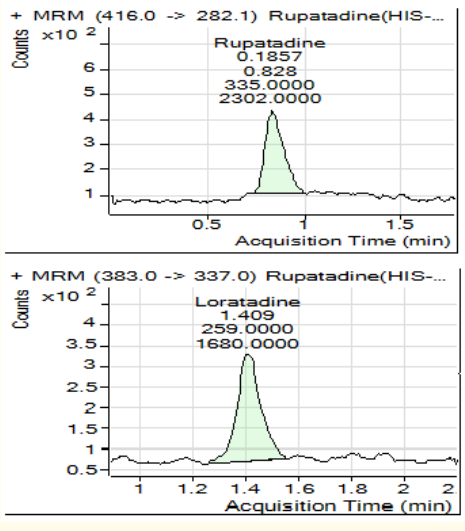


**Figure 2:** Chromatogram - an MRM data of blank plasma spiked with 0.1ng/ml rupatadine and internal standard loratadine.

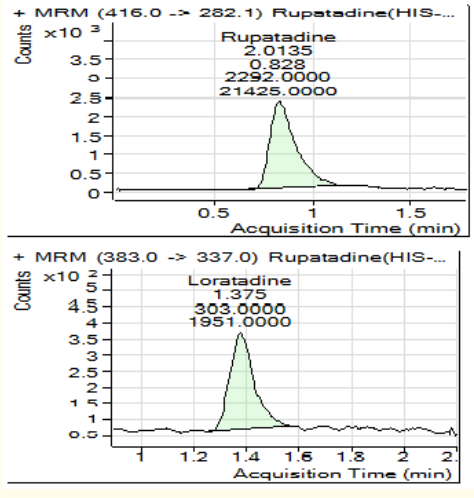


**Figure 3:** Chromatogram - an MRM data of blank plasma spiked with 2ng/ml rupatadine and internal standard loratadine.

| Pharmacokinetic Parameter | Treatment (Mean ± SD) | |
|---|---|---|
| | Generic product | Reference product |
| $C_{max}$ (ng/ml) | 2.652 ± 0.307 | 2.618 ± 0.287 |
| $T_{max}$ (hr) | 0.813 ± 0.169 | 0.802 ± 0.127 |
| $AUC_{0-t}$ (ng.hr/ml) | 16.525 ± 4.341 | 16.679 ± 4.195 |
| $AUC_{0-inf}$ (ng.hr/ml) | 16.620 ± 4.345 | 16.768 ± 4.198 |
| $K_e$ ($hr^{-1}$) | 0.117 ± 0.003 | 0.118 ± 0.003 |
| $t_{(1/2)e}$ (hr) | 5.931 ± 0.174 | 5.902 ± 0.149 |
| MRT (hr) | 7.384 ± 0.509 | 7.323 ± 0.671 |

**Table 1:** Pharmacokinetics for Rupatadine of Generic and Reference Products.

| Pharmacokinetic Parameter | 90% Confidence intervals of parametric means | | |
|---|---|---|---|
| | Point estimate (%) | Lower limit (%) | Upper limit (%) |
| $C_{max}$ | 101.241 | 98.810 | 103.733 |
| $AUC_{0-t}$ | 98.631 | 91.707 | 106.078 |
| $AUC_{0-inf}$ | 98.659 | 91.769 | 106.068 |

**Table 2:** The 90% Confidence Interval for Rupatadine Test and reference Products.

## Conclusion

From the current research, it can be concluded that the developed bioanalytical method for the determination of rupatadine in human plasma was accurate, precise, linear, and valid to be applied for rupatadine detection in bioavailability and bioequivalence studies, clinical trials, therapeutic drug monitoring, efficacy and safety studies of rupatadine. Besides, the results of the bioequivalence study of rupatadine 10mg film coated tablet showed that both generic and reference product are bioequivalent.

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