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A Novel LC/MS/MS Bioanalytical Method for the Determination of Tolterodine and Its Pharmacokinetics Application

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Abstract

Background: Tolterodine is selective for muscarinic receptors of the bladder and is used in the management of urinary frequency, urinary urgency, and urinary incontinence.

Aim: Development of a simple and sensitive bioanalytical method for the determination of tolterodine to investigate their pharmacokinetic parameters in human plasma and its clinical applications, including bioequivalence studies.

Methods: Extracted tolterodine was chromatographed with a mobile phase of ammonium acetate: acetonitrile (20:80 v/v) at flow rate 0.6ml/min, ESI positive mode and m/z 326.1à147.1, 260à183 for tolterodine and the internal standard, respectively. The bioequivalence study was conducted on 24 volunteers, and the pharmacokinetic parameters AUC0-t, AUC0-inf, Cmax, and Tmax were detected for the assessment of the bioequivalence decision of the two products.

Results: The developed bioanalysis technique showed that the average recovery of tolterodine from human plasma was 107.135%, the lower limit of quantitation was 0.01ng/ml, and the correlation coefficient (r2) was 0.9998. The statistical analysis for the pharmacokinetic parameters using the ANOVA test showed a non-significant difference between both drug products included in the study.

Conclusion: The developed LC/MS/MS method is simple, sensitive, and valid for quantification of tolterodine in plasma and is adequate for its clinical pharmacokinetic studies. Moreover, the generic product was found to be bioequivalent to the reference, and both products can be considered interchangeable in medical practice.

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INTRODUCTION

It was reported in a randomized controlled trial of tolterodine compared to oxybutynin that tolterodine 2 mg bid was consistent of equal efficacy to oxybutynin 5 mg tid, and the adverse events were mostly dose-related autonomic nervous system events as dry mouth, which was more frequent and more severe with oxybutynin 5 mg tid than with tolterodine 2 mg bid. Tolterodine caused less dose reduction, patient withdrawal, and adverse events compared with oxybutynin. It was concluded that tolterodine is equally effective as oxybutynin with less intense and less frequent side effects [6].

Clinical study conducted on 26 patients (mean age 8.0±2.2 years) showed that tolterodine remarkably improved the clinical symptoms of overactive bladder in a short time. Besides, tolterodine seems to be as effective as oxybutynin for overactive bladder treatment in children [7].

Tolterodine is marketed in the form of tablets, as a tolterodine tartrate 1 and 2mg under brand name Detrusitol® tablets [2]. Tolterodine is indicated for the treatment of overactive bladder with symptoms of urge urinary incontinence, urgency, and frequency [8], and recommended dosing is 2 mg twice daily, which is reduced, if not tolerated, to 1mg twice daily [2].

The mean values of pharmacokinetic parameters obtained after administration of a single dose of tolterodine 2mg tablet, for Cmax, AUCo-t, and Tmax were 2.1±2.6ng/ml, 7.5±8.6ng.h/ml, and 1 hour respectively [9]. Another study recorded the mean values of Cmax, AUCo-t, and Tmax to be 1.9±1.1ng/ml,6.2±3.4ng.h/ml, and 1 hour respectively [4]; besides, it was reported in a pharmacokinetic study of tolterodine 2mg that the mean values of Cmax, AUCo-t, T1/2, and Tmax were 1.6ng/ml, 6.7ng.h/ml, 2.4h, and 1 to 2 hours respectively [10].

Different analytical methods are investigated and developed for evaluation of tolterodine in pharmaceutical dosage form and biological fluids, including, capillary electrophoresis [11], high-performance liquid chromatography (HPLC) [12–15], ultra-performance liquid chromatography (UPLC) [16], gas chromatography-mass spectrometry (GC–MS) [17]. Liquid chromatography-mass spectrometry (LC-MS) methods have also been reported for the quantitation of tolterodine in human plasma [18–23].

A bioanalytical method used for the determination of tolterodine in biological samples using GC-MS method and plasma sample preparation using solid-phase extraction shows a lower quantitation limit of 0.5ng/ml [24]. A method was used for the determination of tolterodine and its metabolite in biological samples by LC/MS/MS after subjecting plasma sample to liquid-liquid extraction procedure shows a lower quantitation limit of 0.049ng/ml [25].

A sensitive and cost-effective analytical method for determination of tolterodine in biological samples using LC/MS/MS in which the quantitation limit (LLOQ) of 0.02ng/ml and a linear dynamic range of 0.02ng/ml to 5ng/ml [26]. Another study showed a sensitive, specific, and more linear dynamic range bioanalytical method for determination of tolterodine in biological samples by LC/MS/MS with a quantitation limit of 0.025ng/ml and a linear dynamic range of 0.025ng/ml to 10ng/ml [27].

A conducted comparative bioavailability between generic and reference product of tolterodine 2 tablets proved that the method is accurate, precise, selective, and fully validated. The study was conducted on 24 healthy subjects as per protocol. The subjects received one film-coated tablet of generic and reference products in a randomized fashion with a washout period of one week. Twenty-four healthy male volunteers completed the crossover [28].

Analysis of plasma would be done through developing and validating an LC/MS/MS method in compliance with the international guidelines [29]. Pharmacokinetic calculations were performed by the WinNonlin program and statistical analysis (ANOVA) was done using SAS software. Sequence effect was tested, and the 90% confidence intervals for AUC0-t, AUC0-inf, and Cmax were calculated for the ratio or (difference) between treatments, and results showed to be in the limit of 80% to 125% confidence limits [30].

MATERIALS

Chemicals: Deionized purified water of LC/MS/MS grade, methanol HPLC-Gradient (SIGMA Aldrich, Germany), acetonitrile HPLC-Gradient (Scharlab, Spain), formic acid 98-100% EssentQ (Scharlab, Spain), sodium tetraborate decahydrate for analysis ExpertQ (Scharlab, Spain), diethyl ether anhydrous stabilized/HPLC (Fisher Scientific, UK), and dichloromethane for HPLC (Fisher Scientific, UK).

Equipment: Adjustable pipettes (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® plus (Eppendorf, Germany), and LC-MS/MS Agilent 6410B Triple Quad, USA.

METHODS

LC/MS/MS Assay

Chromatographic conditions: In-house developed chromatographic conditions were applied, where the mobile phase composition was ammonium acetate: acetonitrile (20:80v/v), pumped at a flow rate of 0.6ml/min, and the injection volume was set at 10ul. The MS/MS 6410B detector was operated at ESI positive mode, m/z was 326.1→147.1 and 260→183 for tolterodine and propranolol (internal standard), respectively. Fragmentor energy was set at 120 and 90 for tolterodine and propranolol; respectively, with collision energy, was set at 30 and 15 for tolterodine and propranolol, respectively.

Preparation of solutions

Master standard solution: Accurately weighed 10mg of standard tolterodine tartrate were transferred to a 100 ml volumetric flask and about 80 ml methanol was added and sonication was done for 10 minutes, then the volume was completed with methanol to obtain a solution containing 100ug/ml tolterodine tartrate “Solution A” of which 0.05 ml was transferred to a 100 ml volumetric flask which was completed to volume with methanol to obtain a solution of final concentration 50ng/ml “Solution B”.

Master Solution used	Milliliters taken	Final concentration obtained (ng/ml)	Final volume (ml)
"Solution B"	0.02ml	0.1	10
"Solution B"	0.1ml	0.5	10
"Solution B"	0.2ml	1	10
"Solution B"	0.5ml	2.5	10
"Solution B"	1ml	5	10
"Solution B"	2ml	10	10
"Solution B"	4ml	20	10
"Solution B"	6ml	30	10
"Solution B"	8ml	40	10
"Solution B"	10ml	50	10

Working solutions

*All dilutions are done with methanol.

Propranolol standard solution: Accurately weighed 10mg of standard propranolol were transferred to a 100 ml volumetric flask, about 80 ml of methanol was added and sonication was done for 10 minutes, and the volume was completed with methanol to obtain a solution containing 100ug/ml propranolol solution (A) of which 0.02 ml was transferred to a 100ml volumetric flask and completed with methanol to obtain a final concentration 20 ng/ml propranolol solution (B).

Preparation of serial dilution of standard tolterodine in human plasma: The standard samples in human plasma were prepared by transferring a 50 ul aliquot of the working standard solutions of tolterodine at concentrations ranging from 0.1 to 50 ng/ml to a centrifuge tube containing 500 ul of blank human plasma.

Sample preparation: The collected plasma samples of subjects (500 ul) were transferred into centrifuge test tubes and 50 ul of propranolol working solution of concentration 20 ng/ml and vortex-mix for approximately t minute followed by addition of 3ml of diethyl ether/dichloromethane 70:30 v/v) and vortex-mix for approximately 1 to 2 minutes. Centrifugation of samples was done at 3500 r.p.m. for 5 minutes, and the clear organic layer was transferred to a clean test tube and evaporated till dryness; the residue was reconstituted with 200ul mobile phase and transferred to insert vial for quantitation on LC/MS/MS.

Quantitation: The unknown sample concentration of volunteer human plasma was calculated from the following formula: $Y = aX + b$, where, Y: is the peak area ratio, X: is the unknown Concentration of Tolterodine in human plasma samples, a: is the slope of the calibration curve, and b: is the Y-Intercept.

Clinical Application as Bioequivalence Study

Study ethics: This study was conducted per the international conference of harmonization (ICH) and good clinical practice (GCP) guidelines adopted by the European agency for the evaluation of medicinal products (EMEA) [31], and after ethics committee approval on the bioequivalence study protocol of tolterodine 2 mg film-coated tablets (Study Code: NIL-NCPC-BCS-1115/0217). Essential documents and records were all archived according to drug research center (DRC) internal procedures of authorized direct access.

Written informed consent was 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 did not want to.

Clinical Investigator, study director (principal investigator), licensed physicians responsible for physical examination and following-up of the subjects for the 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, the subjects should be without a known history of alcohol or drug abuse problems and should preferably be non-smokers.

Exclusion criteria: Known hypersensitivity to the drug, gastrointestinal diseases, autoimmune 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, a subject which have taken any medication (Rx or OTC) less than two weeks of the trials starting date, a subject who has 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 who 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 did not have any acute or chronic gastrointestinal, cardiac, vascular, hepatic, or renal disease. The concurrent medication was not allowed during the time course of the study, and meals, beverages drink, coffee, or tea are not allowed for four hours after study dose administration. At 12:00, they received a standard meal, and at 16:00, another meal.

Study design: This study was constructed in a two-way crossover design to compare the bioavailability of generic versus reference products of tolterodine in 24 healthy adults, male volunteers under fasting conditions with a washout period one week between dosing. The number and disposition of the blood collections, as well as the washout period, were designed with respect to pharmacokinetic parameters of tolterodine.

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

Analysis of plasma samples: An LC-MS/MS technique was used for the quantitation of tolterodine in human plasma, where, all withdrawn volunteer samples were analyzed.

Pharmacokinetic calculations: The following pharmacokinetic parameters (variables) of tolterodine were assessed; maximum plasma concentration (C_{max}), the time point of maximum plasma concentration (t_{max}), the half-life of drug elimination ($t_{1/2e}$), terminal rate of elimination (K_e), and the 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 tolterodine within the prescribed 90% confidence interval of 80 to 125% for AUC_{0-t}, AUC_{0-inf}, and C_{max}.

Results

Bioanalytical Method Validation

Chromatograms of tolterodine: Tolterodine and its internal standard were well separated, and their retention time was 1.9 and 1.8 min respectively, obtained peaks were sharp, symmetrical with good baseline resolution and minimum tailing, thus facilitating the accurate measurement of the peak area. The in-house developed chromatographic conditions were in accordance with those published in the literature [25-27], with some modification in extraction and chromatographic conditions.

Linearity, precision and accuracy: Peak area ratios of varying amounts of tolterodine in human plasma in the range of 0.01 to 5 ng/ml were highly linear with a correlation coefficient (r^2) of 0.9998, and the average results of interday coefficient of variation C.V.% was 1.990%. Accuracy and precision were assessed on at within-day, and between-day basis at three drug concentrations in the range of expected concentrations, the results of intra-day and inter-day accuracy showed an average recovery percentage of 101.117% and 101.285%, respectively, with an average C.V. % of 1.990%. The results of stability in human plasma showed that the average recovery of tolterodine was greater than 95%, ensuring that tolterodine is stable in the studied conditions.

Bioequivalence Study

Clinical observation: The drug was well tolerated by 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 bioequivance: The mean values of C_{max}, t_{max}, $t_{1/2e}$, AUC_{0-t}, and $AUC_{0-\infty}$ were 2.600 ± 0.438ng/ml and 2.583 ± 0.358ng/ml, 0.979 ± 0.275h and 0.979 ± 0.244h, 2.874 ± 0.152h and 2.881 ± 0.128h, 10.729 ± 2.561ng.h/ml and 10.699 ± 3.552ng.h/ml, 10.781 ± 2.559ng.h/ml and 10.751 ± 3.554 ng.h/ml for the generic and reference products respectively.

Statistical analysis: The results of two-way ANOVA for C_{max}, $AUC_{0-t,}$ and $AUC_{0-inf,}$ showed that there was no significant difference between generic and reference product, and the point estimate (%) results for C_{max}, AUC_{0-t}, AUC_{0-inf} were 100.169, 102.002, and 101.993%, respectively. The 90% confidence intervals of parametric means of C_{max}, AUC_{0-t}, and AUC_{0-inf} were 95.838 to 104.695%, 95.667 to 108.756%, and 95.681 to 108.721%, respectively.

DISCUSSION

The LC/MS/MS method used in this study was of excellent sensitivity, specificity, precision, and accuracy. The calibration curve was linear over the concentration range of 0.01 to 5ng/ml, and r^2 was equal to 0.9998, which is per FDA guidelines [29], and so it could be used for pharmacokinetic and bioavailability studies of tolterodine.

Literature publication showed an analytical method developed for the determination of tolterodine in human plasma. The method was validated, simple, rapid, and sensitive high-performance liquid chromatography analytical method using LC–MS/MS to quantitate tolterodine and its major metabolites, 5-hydroxymethyltolterodine (5-HMT) and N-dealkyltolterodine (NDT) in plasma. Liquid–Liquid extraction with methyl t-butyl ether was used, and chromatographic separation of the three analytes was achieved using a reversed-phase Phenyl-hexyl column (100 × 2.0 mm, 3 µm particles) with a mobile phase of 10 mM ammonium formate buffer pH 3.5: methanol (10:90, v/v). Detection mode was set on ESI positive ion mode. Calibration curves were linear over a range of 0.025–10 ng/ml for tolterodine and 5-HMT, and 0.05–10 ng/ml for NDT. The lower limit of quantifications using 200 µl of human plasma was 0.025 ng/ml for tolterodine and 5-HMT, and 0.05 ng/ml for NDT [32].

The in-house developed chromatographic conditions showed to have LLOQ of 0.01ng/ml which is better than the reported published literature analytical methods LOQs of 0.049ng/ml, 0.02ng/ml, and 0.025ng/ml [24-27, 32].

The use of the internal standard in bioanalytical is an important issue to ensure the accuracy and validity of target drug quantification and compensate for any variation due to sample processing and detector response alterations. In the current study, propranolol was used as an internal standard reported in the literature [25].

The results of tolterodine pharmacokinetic parameters obtained in this research were in accordance with that reported literature, including $T_{max,}$ which was found to be ranged from 1 to 2 hours, C_{max} ranged from 1.6ng/ml to 2.1ng/ml, and $T_{1/2}$ were 2.4 h on average [9-10].

In a bioequivalence study, a 90% confidence interval of 80 to 125% 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 point estimate (%) results for C_{max}, AUC_{0-t}, AUC_{0-inf} were 100.169, 102.002, and 101.993% respectively.

The 90% confidence intervals of parametric means of C_{max}, AUC_{0-t}, and

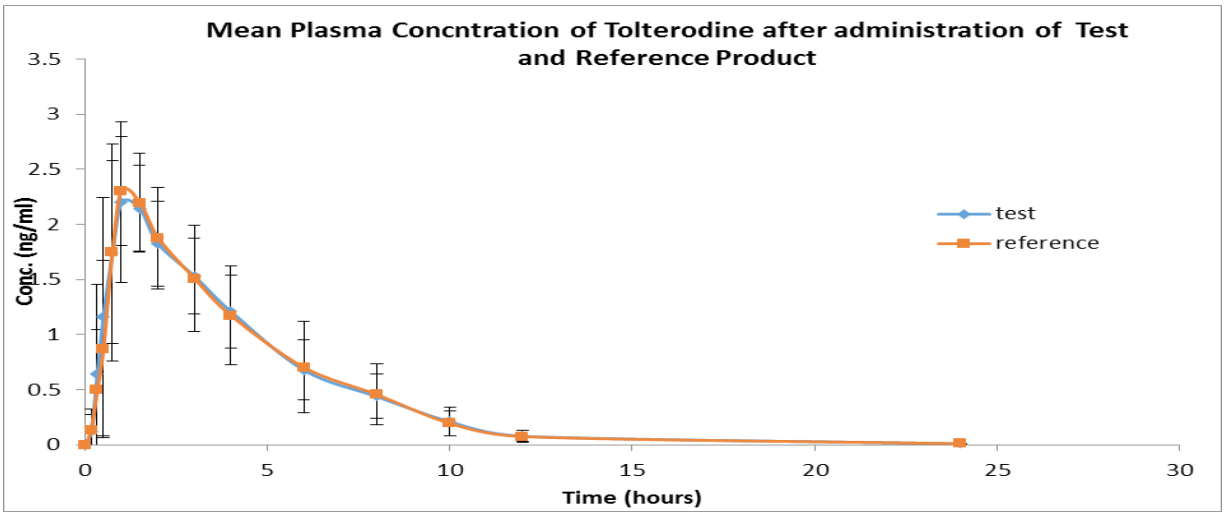
Table 1: Pharmacokinetics of Generic and Reference Tolterodine Products

Pharmacokinetic Parameter	Treatment (Mean ± SD)	
	Generic product	Reference product
C_{max} (ng/ml)	2.600±0.438	2.583±0.358
T_{max} (h)	0.979±0.275	0.979±0.244
AUC_{0-t} (ng.h/ml)	10.729±2.561	10.699±3.552
AUC_{0-inf} (ng.h/ml)	10.781±2.559	10.751±3.554
K_e (hr^{-1})	0.242±0.013	0.241±0.011
$t_{(1/2)e}$ (h)	2.874±0.152	2.881±0.128
MRT (h)	4.342±0.445	4.265±0.427

Table 2: The 90 % C.I for Generic and Reference Tolterodine Products

Pharmacokinetic Parameter	90% Confidence Intervals of Parametric Means		
	Point estimate (%)	Lower limit (%)	Upper limit (%)
C_{max} (ng/ml)	100.169	95.838	104.695
AUC_{0-t} (ng.hr/ml)	102.002	95.667	108.756
AUC_{0-inf} (ng.hr/ml)	101.993	95.681	108.721

Figure 1: Plasma concentration (Mean ± S.D.) of Tolterodine following single dose administration of generic and reference products



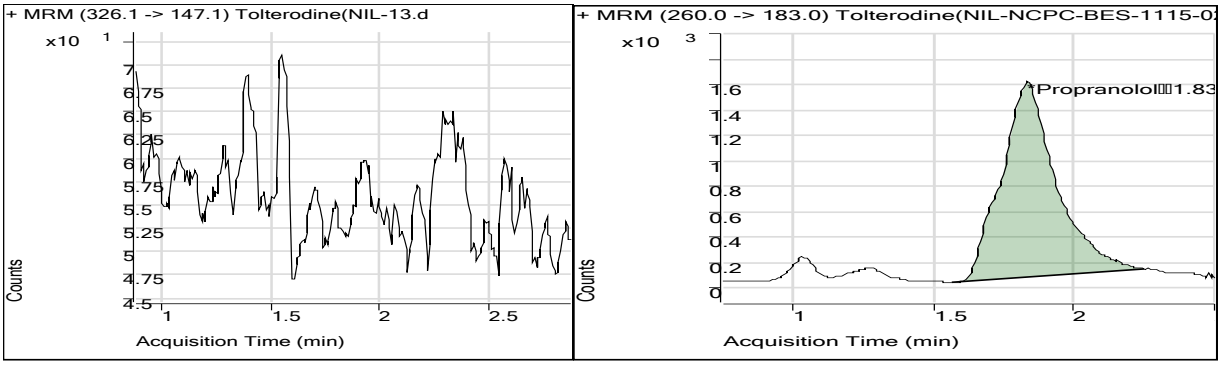


Figure 2: Chromatogram - an MRM data of blank plasma spiked with Propranolol.

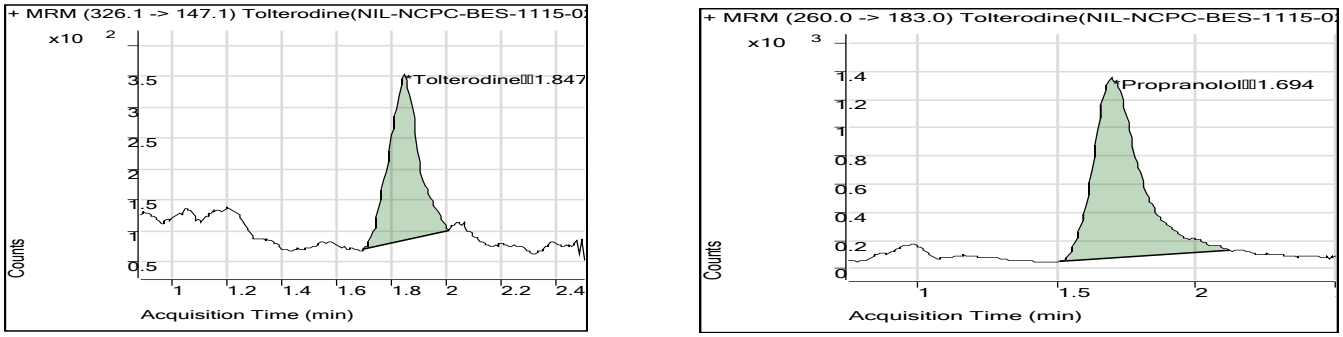


Figure 3: Chromatogram - representing an MRM data of blank plasma spiked with 0.01ng/ml Tolterodine and Propranolol.

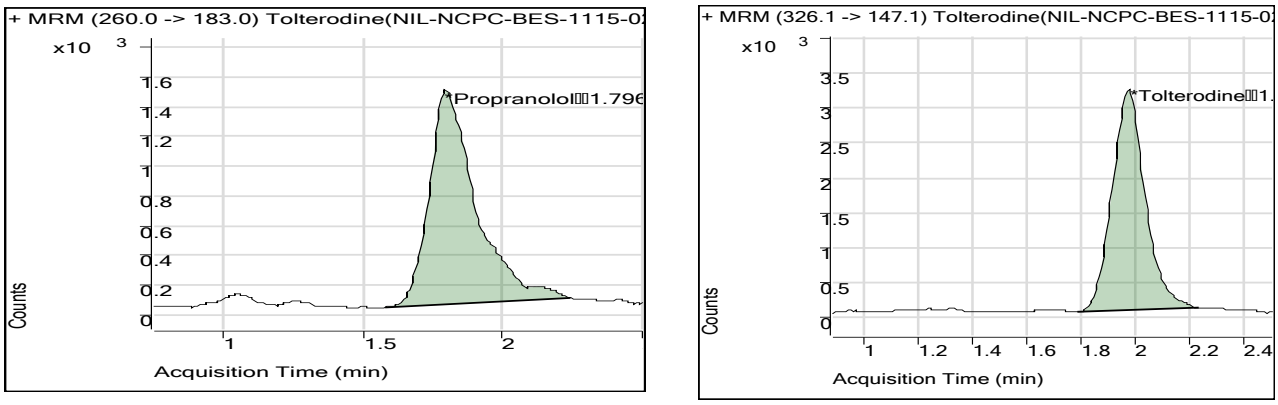


Figure 4: Chromatogram - representing an MRM data of blank plasma spiked with 2ng/ml Tolterodine and Propranolol.

$AUC_{0\text{-}inf}$ were 95.838 to 104.695%, 95.667 to 108.756%, and 95.681 to 108.721%, respectively thus providing a 90% confidence intervals limits lying within FDA acceptance limits of (80 to 125%) [30].

CONCLUSION

From the obtained results, it can be concluded that the bioanalytical method developed for the determination of tolterodine in human plasma is fully validated and could be used in clinical applications including bioavailability and bioequivalence studies, clinical trials, therapeutic monitoring, efficacy, and safety studies. The method showed linearity (r2) of 0.9998, and linearity range of 0.01 to 5 ng/ml. Besides, accuracy and precsion results showed to be within acceptance limits. The statistical analysis of the obtained pharmacokinetic parameters of both generic and reference products showed a non-significant difference and lying within FDA acceptance limits of (80 to 125%), thus the decision of being bioequivalent was concluded.

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