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Research Article

Determination of Donepezil in Human Plasma and its Clinical Applications

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Abstract

Background: Alzheimer's disease is a common form of memory loss of high incidence in geriatric populations. Donepezil is a medication known to improve the cognitive functions, perception, and behavior.

Aim: Development of a bio-analytical method for rapid quantification of donepezil in biological fluids and its application in pharmacokinetics and bioavailability studies, clinical trials, and monitor its therapeutic levels to help attaining effective clinical results in management of Alzheimer's disease.

Methods: Donepezil was extracted from plasma samples and chromatographed with eluting solvent consisting of 0.1% formic acid: acetonitrile: methanol (20:40:40) v/v/v at flow rate of 0.52ml/min, ESI positive mode, and m/z 380.3 → 91, 290.2 → 115 for donepezil and desbenzyl donepezil as internal standard respectively. As an application of the validated developed bioanalytical method, a comparative bioavailability study of donepezil 10 mg film coated tablets generic product versus reference product was conducted in a crossover design involving 24 volunteers. The criteria used to assess bioequivalence of the two products were AUC 0-72, C_{max}, and T_{max}.

Results: The average recovery of donepezil from human plasma was 86.251% with limit of quantitation of 0.1ng/ml and the correlation coefficient (r2) obtained was 0.9999, moreover, statistical analysis (ANOVA) of the measured parameters showed that there was no significance between the two products.

Conclusion: The developed bioanalytical LC/MS/MS method was valid for donepezil quantification in human plasma and is suitable for application in pharmacokinetic studies and therapeutic monitoring of donepezil in management of Alzheimer's disease to ensure effective therapeutic drug levels and avoid potential undesired adverse events.

Keywords: Donepezil; Alzheimer's Disease; Dementia; LC/MS/MS; Validation; Liquid-liquid Extraction

Introduction

Donepezil is a piperidine derivative working through reversible and selective inhibitor of acetylcholinesterase in a similar action to those of neostigmine which is highly selective for the CNS and is used for the symptomatic treatment of dementia in Alzheimer's disease [1].

Dementia is a progressive disease that damages the brain and ultimately becomes severe enough to interfere with daily activities, also, Alzheimer's disease (AD) considered as a common form of memory loss [2-4]. There is an evidence from naturalistic trials as well as observational studies that donepezil is effective against

the three main domains of Alzheimer's disease symptoms, which are functional ability, behavior, and perception. Besides, it is also indicated that improved outcomes are supported by long term management of Alzheimer's disease and maintenance of cognitive benefits [5].

It was reported that donepezil provided a remarkable advantages compared with placebo regarding cognitive functions, effectiveness and safety in both Alzheimer's disease and vascular dementia patients in regarding their co-morbidities and concomitant medications [6], moreover, its co-administration with memantine results in an additive clinical benefits of about 50% or higher compared to individual donepezil therapy through a time course of six-month treatment [7].

Donepezil hydrochloride is marketed as 5mg and 10 mg film coated tablets under brand name of Aricept® [8] which is indicated for the relief of mild to moderate symptoms of dementia in Alzheimer's disease with an initial dose of 5 mg once per day for one month and increased to 10 mg daily given at bedtime [9].

Concerning the pharmacokinetics of donepezil, it was found that after single dose administration of donepezil hydrochloride 10mg film coated tablet, the mean values of C_{max}, AUC_{o-inf}, $T_{1/2}$, and median T_{max} was 26.35 ± 6.51 ng/ml, 1108.44 ± 268.52 ng.hr/ml, 72.81 ± 11.78 hr. 2 hours (range from 1 to 4 hours) respectively [10]. Different analytical methods are developed for the assay of donepezil in biological fluids; including HPLC equipped with an ultraviolet (UV) [11,12], fluorescence (FL) [13,14], and mass spectrometric detector [15,16].

Various analytical methods were used for the quantitation of donepezil in biological specimen including; an HPLC-UV method with a lower quantitation limit (LLOQ) of 3 ng/ml [17], an HPLC-FL method with LLOQ of 5 ng/ml [18] and LC/MS/MS different methods with LLOQ of 0.5 ng/ml [19] and LLOQ of 0.1 ng/ml and a linear dynamic range of 0.2 ng/ml to 20 ng/ml [20].

Moreover, a sensitive LC/MS-TOF assay was developed for the quantification of donepezil in biological sample using liquid-liquid extraction, multiple reaction monitoring (MRM) was based on m/z 380$[M + H]^+$ for donepezil, m/z 383$[M + H]^+$ for loratadine as an internal standard with LLOQ 0.1 ng/ml with linearity range of 0.1 to 15 ng/mL [21]. Another method used mass to charge ratios, m/z 380 → 91 for donepezil and m/z 384 → 253 for quetiapine as internal standard with LLOQ of 0.1 ng/mL and linearity range of 0.1 to 42 ng/mL [22].

The objective of this study was to introduce a valid bioanalytical method for the determination of donepezil in human plasma and its application in pharmacokinetic and bioavailability studies, clinical trials and therapeutic monitoring of donepezil in patients to ensure safety and efficacy in management of alzheimer's disease.

To examine the efficiency of the developed bioanalytical method for the quantitation of donepezil in biological fluids in this study, a comparative bioavailability study of donepezil generic versus reference products was conducted according to international guidelines, where, the protocol called for 28 to 24 healthy volunteers with a washout period of three weeks [23].

The developed validated LC/MS/MS method was in compliance with the international guidelines for the bioanalysis of plasma samples [24], and the pharmacokinetic calculations were done using WinNonLin program. Besides, statistical analysis (ANOVA) was performed using SAS software and the 90% C.I. limits for the ratio between generic and reference products for the obtained pharmacokinetic parameters of AUC_{0-72} and C_{max} were calculated to investigate its compliance with range of 80% to 125% confidence limits [25].

Methods

LC/MS/MS analytical method

Mass parameters and chromatography

The method was developed in-house as follow: eluting solvent composition is 0.1% formic acid: acetonitrile: methanol (20:40:40) v/v/v, the flow rate was set at 0.52ml/min with injection volume set at 1.4 ul., the MS/MS 6410B detector was operated at ESI positive mode, m/z was 380.3 → 91, 290.2 → 115 for donepezil and desbenzyl donepezil as internal standard respectively. The fragmentor energy was set at 140 for donepezil and 145 for desbenzyl donepezil, and collision energy was set at 37 for donepezil, 45 for desbenzyl donepezil.

Preparation of solutions

Donepezil standard solution

An accurately weighed 11.67mg of standard donepezil HCl equivalent to 10 mg of donepezil was transferred into a 100 ml volumetric flask, 80 ml of methyl alcohol was added and sonicated for 10 minutes, then completed to volume with methyl alcohol, to obtain a solution with final concentration of 100ug/ml donepezil (Solution A) of which 0.5ml was transferred to a 100 ml volumetric flask and completed to volume with methyl alcohol to obtain final concentration of 500 ng/ml donepezil (Solution B).

Working solutions

Master Solution used	Mililitres taken	Final concentration obtained (ng/ml)	Final volume (ml)
"Solution B"	0.02ml	1	10
"Solution B"	0.1ml	5	10
"Solution B"	0.2ml	10	10
"Solution B"	0.5ml	25	10
"Solution B"	1ml	50	10
"Solution B"	2ml	100	10
"Solution B"	4ml	200	10
"Solution B"	8ml	400	10

Table: All dilutions are done with methanol.

Desbenzyl donepezil hydrochloride standard solution

An accurately weighed 10 mg of desbenzyl donepezil hydrochloride standard was transferred into a 100 ml volumetric flask and about 80 ml of methanol was added and was sonicated for 10 minutes, then volume was completed with methanol to obtain final concentration solution of 100 ug/ml desbenzyl donepezil hydrochloride, 20 ml of which was transfer to 100 ml volumetric flask and completed to volume with methanol to obtain 20 ug/ml desbenzyl donepezil hydrochloride.

Preparation of serial dilutions of standard donepezil in human plasma

Serial dilutions of standard donepezil in human plasma were prepared by transferring 20 ul aliquot of donepezil of concentrations ranging from 1 to 400 ng/ml to a centrifuge tubes containing 200ul of blank plasma.

Preparation of plasma samples

The collected plasma samples of subjects, 200 ul, were transferred into centrifuge test tubes and 20 ul of desbenzyl donepezil working solution 20 ug/ml was added, then vortex-mix were for 1 minute followed by addition of 2 ml of cyclohexane:dichloromethane 4:1 v/v and vortex-mix for nearly 1 to 2 minutes. The samples were centrifuged at 3500rpm for 5 minutes at 4°C, the clear organic supernatant layer was transferred to a clean test tube and evaporate till dryness, 200ul mobile phase were used to reconstitute dry residue and transferred to insert vial for analysis by LC/MS/MS.

Quantitation

The unknown plasma sample concentration was calculated as per formula: $Y = aX + b$. Where Y is the response ratio, X is the unknown concentration of drug in plasma samples, a is the calibration slope, b is the Y-Intercept

Application on bioequivalence study

Ethics

This study was conducted in accordance with ICH and GCP guidelines adopted by (EMEA) and after Ethics Committee approval on donepezil 10mg tablet bioequivalence study protocol (Study Code: NOR-TABT-BES-1116/0237). All documents and records were archived according to Drug Research Center internal procedures.

The participant, clinical investigator, and other responsible persons signed a written informed consents. Before starting of screening step, all study aspects where discussed with participants. There were no any obligations on volunteers to continue the bioequivalence study if they didn't want to.

Principal investigator and clinical investigator were responsible for supervising all study procedures. Licensed physicians were responsible for physical examination and following-up of subjects for measurement of vital signs, including blood pressure, pulse rate, body temperature, respiratory rate, and monitoring appearance of any side or adverse events throughout the study. Blood sampling were performed by registered nurses.

Inclusion criteria

Subjects age within 18 to 55 years and calculated BMI within normal acceptable limits. Normal physiological examination and laboratory data were within normal limits. Subjects of no alcoholic

or drug abusers and shouldn't have any known history for both, of no clinical study contribution history. Non-smoker subject was preferred over smoker subject and if smoker, this should be not more than 8 cigarettes per day.

Exclusion criteria

Any donepezil hypersensitivity, GIT problems, hematological abnormalities, kidney diseases, auto-immune diseases, CVS diseases, diabetics, hepatic disease, respiratory diseases, history of alcohol intake, and drug abuse, positive HIV, abnormal laboratory values, subject administered any medication less than two weeks of the study starting date, blood donation or participation in clinical studies that requires more than 500 ml of blood loss within month and half preceding starting date of the bioequivalence study.

Subjects

Twenty-four healthy adult subjects participated in the bioequivalence study and were subjected to general physical examination, neurological examination, clinical urine tests and blood analysis. The selected subjects had no history of drug or alcohol abuse and have no 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 11:00 a.m. they received a standard breakfast meal followed by a lunch meal at 3:00 p.m.

Study design

The design of this study was a single-center open-label randomized single-dose two-way crossover design to compare the bioavailability of generic versus reference donepezil 10 mg film coated tablet in 24 healthy male adults under fasting conditions with a washout period of three weeks 21 days.

Collection of sample

The number and disposition of the blood collections as well as the wash out period were designed with respect to pharmacokinetic parameters of donepezil.

The number of blood collections for drug analysis was 17 samples in each period, 5ml per sample collected at the following intervals: 0 (directly prior to dosing), 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12, 24, 48, and 72 hr after dose administration in tubes containing anticoagulant EDTA disodium and centrifuged at approximately 4000 r.p.m. for 10 minutes and plasma samples were separated in a 5 ml-plastic Wassermann tube. The collected samples were stored at a -80°C until analysis. The study code, subject number, study period, time interval was recorded on the tubes. Total blood amount withdrawn during the whole study did not exceed 170 ml.

Analysis of samples

Determination of donepezil in plasma samples of the participants was performed by LC-MS/MS using the developed bioanalytical method was validated according to the international guidelines.

Calculation of the pharmacokinetics parameters

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

Statistical analysis

Statistical analysis of the calculated pharmacokinetic data was performed using statistical computerized program SAS software for determination of analysis of variance (ANOVA). Bioequivalence could be demonstrated for donepezil within the prescribed 90% confidence limit of 80.00% to 125.00% for AUC_{0-72}, and C_{max} with respect to the parametric method on Ln-transformed data.

Clinical observation (Safety and Tolerability)

Subject medical histories, physical examination and laboratory reports, and all incidents of possible adverse reactions to the study formulations were reported.

Measurement of blood pressure and heart rate

Blood pressure systolic/diastolic and pulse rate measurements before dosing and at regular intervals (at 2, 4, 6, and 10 hours) after drug administration were included in tolerability assessments. A 120/80 mmHg blood pressure reading and 50 to 100 beats per minute resting heart rate are considered normal.

Results

Validation of the LC/MS/MS analytical procedure

Chromatograms of donepezil

It is apparent from figure 1-3 that donepezil and desbenzyl donepezil were well separated and their retention time was 1 minute, sharp and symmetrical peaks showed a good baseline with minimum tailing thus facilitating the accurate measurement of the peak response. The in house developed chromatographic conditions was in accordance with published literature [19-22] with some modification in extraction procedure and chromatographic conditions.

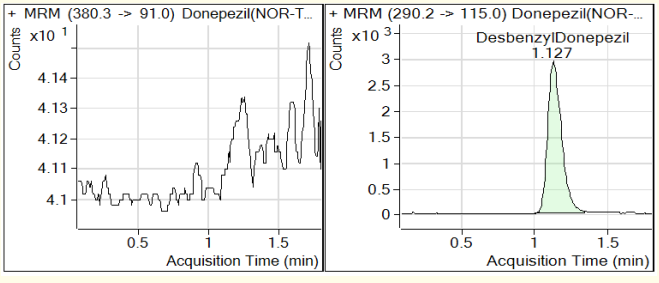

Figure 1: Chromatogram - an MRM data of blank plasma spiked with internal standard desbenzyl donepezil.

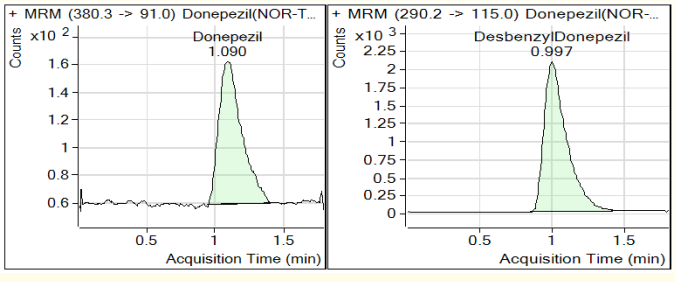

Figure 2: Chromatogram - an MRM data of blank plasma spiked with 0.1 ng/ml donepezil and internal standard desbenzyl donepezil.

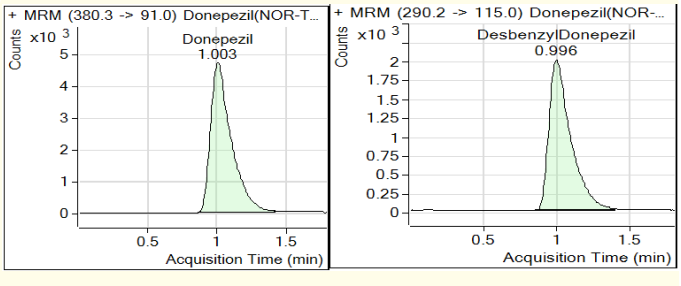

Figure 3: Chromatogram - an MRM data of blank plasma spiked with 20 ng/ml donepezil and internal standard desbenzyl donepezil.

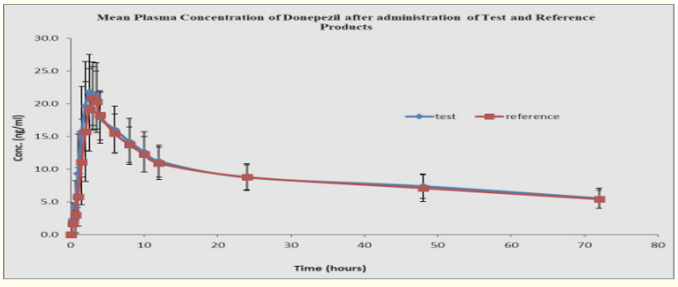

Figure 4: Plasma concentration (Mean ± S.D.) of donepezil following single dose administration of donepezil 10 mg film coated tablets of generic and reference products.

Linearity, precision and accuracy

The peak area ratios of serial dilutions of donepezil in human plasma of concentrations ranging from 0.1 to 400 ng/ml was highly linear with r^2 of 0.9999. The C.V.% of the average results of inter-day variation was 1.888% in accordance with FDA Guidelines [24] which strengthen the possibility of its application in pharmacokinetics and bioavailability studies of donepezil.

Accuracy and precision were assessed on within-day and between-day basis at three levels of drug concentrations at the expected range. Moreover, the results of intra-day inter-day accuracy showed an average recovery percentage of 96.417% and 99.339% respectively. The results of freeze-thaw, short term and long term stability in human plasma showed that the average recovery of donepezil was greater than 95% providing that both targeting drug and internal standard were stable in the studied conditions.

Comparative bioavailability study

Clinical observation (Safety and tolerability)

The drug was to some extent tolerated by most of the participants with development of some documented side effects as headache, nausea, gastrointestinal upset and drowsiness occurred. But, no treatment related adverse events or laboratory abnormalities were observed. Blood sampling during the whole study was performed at the proper time.

During phase I of the study subjects 1, 10, 16, and 26 complained from headache, subjects 1, 14, 18, and 23 complained from nausea, subjects 14, 16, and 23 complained from drowsiness.

During phase II of the study subject 26 complained from headache, subjects 14, and 18 complained from nausea, subjects 14, and 18 complained from drowsiness. No subjects withdrew from the study for any reason attributable to drug side effects.

Pharmacokinetic data and assessment of bioequivalence

Results of pharmacokinetic parameters presented in table 1 and 2 showed that the mean values for C_{max} was 25.384 ± 4.124ng/ml and 25.251 ± 3.279 ng/ml, t_{max} was 2.771 ± 0.571h and 2.917 ± 0.565h, $t_{1/2e}$ 69.068 ± 16.528 and 69.355 ± 18.786h, and AUC_{0-72} 644.302 ± 119.442 ng.h/ml and 622.808 ± 125.908 ng.h/ml for generic and reference products respectively which were in accordance with those reported in the literature [10].

Subject	T_{max} (hr.)	C_{max} (ng/ml)	AUC_{0-72} (ng.hr/ml)	K_{el} (hr^{-1})	$T_{1/2}$ (hr)
Mean	2.917	25.251	622.808	0.011	69.355
CV%	19.360	12.985	20.216	26.863	27.087
Range (Median)	2.00-4.00 (3.000)	20.032-31.233 (25.558)	362.172-800.712 (650.056)	0.007-0.017 (0.011)	41.396-97.859 (63.321)

Table 1: Pharmacokinetics parameters of donepezil following administration of reference product.

Subject	T_{max} (hr.)	C_{max} (ng/ml)	AUC_{0-72} (ng.hr/ml)	K_{el} (hr^{-1})	$T_{1/2}$ (hr)
Mean	2.771	25.384	644.302	0.011	69.068
CV%	20.595	16.248	18.538	27.773	23.930
Range (Median)	2.00-3.50 (2.500)	15.281-33.254 (25.791)	445.287-911.473 (617.326)	0.007-0.017 (0.010)	41.893-95.826 (72.565)

Table 2: Pharmacokinetics parameters of donepezil following administration of generic product.

Statistical analysis

Two-way ANOVA was performed for C_{max}, T_{max}, and AUC_{0-72} of the two products, also, 90% confidence limit of 80.00% to 125.00% for AUC_{0-72} and C_{max} with respect to the parametric method on Ln-transformed data should be fulfilled. In this bioequivalence study the point estimate (%) results for C_{max}, AUC_{0-72}, were 99.977% and 104.015% respectively and the 90% confidence intervals of parametric means of C_{max}, AUC_{0-72} were 94.536% to 105.731%, 96.861% to 111.697% respectively, (Table 3) thus, providing a 90% confidence intervals limits within FDA acceptance limits [25].

Blood pressure and pulse rate

The reported measurements of blood pressure and pulse rate were all approaching normal levels and within the safe limits (Figure 5 and 6).

Pharmacokinetic Parameter	90% C.I. of parametric means		
	Point estimate (%)	Lower limit (%)	Upper limit (%)
C_{max} (ng/ml)	99.977	94.536	105.731
AUC_{0-72} (ng.hr/ml)	104.015	96.861	111.697

Table 3: 90% C.I. for generic and reference products.

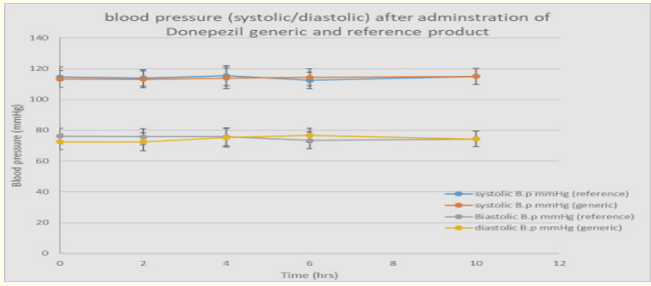

Figure 5: Blood pressure systolic/diastolic measurement (Mean ± SD) after single oral administration of donepezil generic and reference products.

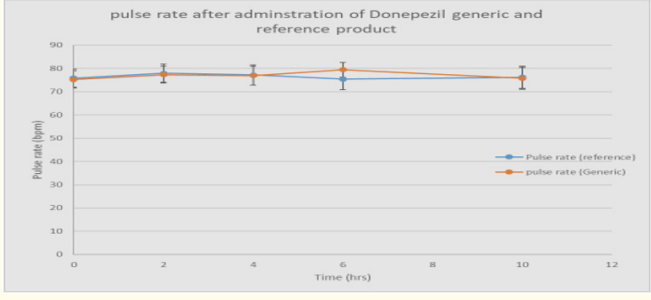

Figure 6: Pulse rate (Mean ± SD) after single oral administration of donepezil generic and reference products.

It is clear from the blood pressure results represented in figure 7 for the generic product, that all approaches normal levels, as the reported mean values of systolic blood pressure were 113, 113, 114, 114, 115 mmHg and 73, 73, 75, 77, 74 mmHg for diastolic blood pressure at Zero (predose), 2, 4, 6, and 10 hours of drug administration respectively.

On the other hand, concerning the reference product, mean values of systolic blood pressure were 115, 114, 115, 113, 115 mmHg and 76, 76, 76, 73, 74 mmHg for diastolic blood pressure at Zero (predose), 2, 4, 6 and 10 hours of drug administration respectively (Figure 8).

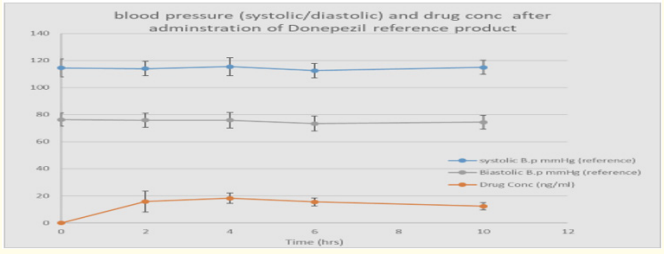

Figure 7: Blood pressure systolic/diastolic measurements corresponding to donepezil plasma concentration (Mean ± S.D.) after single oral administration of reference product.

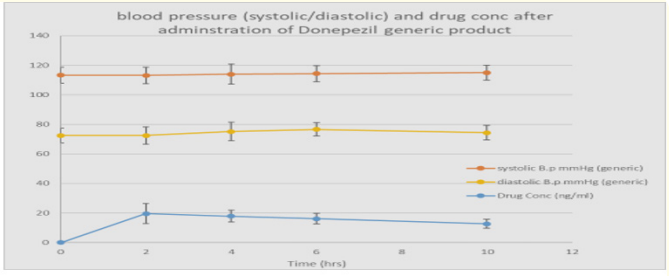

Figure 8: Blood pressure systolic/diastolic measurements and corresponding donepezil plasma concentrations (Mean ± SD) after single oral administration of generic product.

Discussion

The developed bioanalytical method proved to be sensitive, specific, precise and accurate, showing linearity in the range of 0.1 to 400 ng/ml with r^2 of 0.9999, and thus, in compliance with the FDA Guidelines [24], which could be applied in bioavailability and clinical studies, clinical trials, therapeutic monitoring of donepezil to assure safety and efficacy in Alzheimer's disease management.

A published literature UPLC-MS/MS method was developed and validated for the quantification of donepezil in human plasma. Liquid-liquid extraction method was used to extract donepezil and donepezil-D4 (internal standard) from plasma by using a mixture of hexane and ethyl acetate (70:30 v/v). analysis was performed on Thermo Hypersil Gold C_{18} column with mobile phase of 5% acetic acid in 20 mM ammonium acetate buffer (pH 3.3) and acetonitrile 60:40 (v:v) in isocratic mode, at a flow rate of 0.3 mL/min. Injection volume was 3 µL, and the total run time was 3 min. Method accuracy ranged from 98.0% to 110.0%, and precision was below 8%. LOQ was 0.1 ng/ml. The method was successfully applied to donepezil quantification in human plasma [26].

The in house developed chromatographic conditions was in accordance with published literature [19-22,26] with some modification in the extraction procedure and chromatographic conditions and using desbenzyl donepezil as a structurally related internal standard. It is worthy to mention that, the development of an accurate and precise bioanalytical assay was important for ensuring accurate and precise therapeutic monitoring and testing the validity of generic products for commercial use with targeted clinical outcomes.

Donepezil showed better clinical efficacy than memantin at a treatment time point, and similar efficacy at another time point, this was found in a clinical study, in which donepezil showed trends toward a better daily living improvement after 8-week treatment compared to memantine. On the other hand, there was no difference in any outcome between the two drugs after 16-weeks of treatment, where significant improvement in the MMSE and Blessed-Roth scores occurred, and global function was not changed by donepezil and memantine [27].

When comparing donepezil to tacrine (the first approved AChEI) in Chinese patients, donepezil showed equivalent improvement in cognitive function and activities of daily living, and better tolerability and safety in patients with mild to moderate Alzheimer disease [28].

The importance of donepezil therapeutic drug monitoring is that it proved to be effective against three main domains of Alzheimer's disease symptoms, which are functional ability, behavior, and perception [5].

Gastro-intestinal side effects are the most common. These include diarrhea, vomiting, and nausea. Dizziness, headaches, muscle cramps, insomnia, anorexia, and fatigue are other common side effects. In most patients, these side effects are mild and transient, lasting up to three weeks and are usually resolved even with continued use [29,30], suggesting that donepezil is tolerable and safe.

The determined pharmacokinetics parameters of donepezil, in the current study, by using the developed validated bioassay method were in accordance with those reported in the literature [10] which might be an evidence for the successful application of this method for clinical studies used for evaluation of donepezil outcomes in patients.

The reported results of the conducted comparative bioavailability study showed that the 90% confidence limit lied in the range 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 and therefore conclusion of that both the generic and reference products of donepezil were bioequivalent as per FDA acceptance limits (80 % to 125%) [25].

Conclusion

The developed bioanalytical method for the quantitation of donepezil in plasma is fully validated and can be applied for bioavailability studies, clinical trials, therapeutic drug monitoring. Moreover, the results of the comparative bioavailability study showed that both the generic and the reference products were as bioequivalent.

Donepezil is an effective drug treatment for management of Alzheimer's disease and its therapeutic monitoring is an important approach for meeting the required therapeutic goals as a consequence of monitoring patients' drug levels to avoid subtherapeutic or toxic drug levels.

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