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Quantitative determination of donepezil in human plasma by liquid chromatography/tandem mass spectrometry employing an automated liquid–liquid extraction based on 96-well format plates Application to a bioequivalence study

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Abstract

An automated high-throughput liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed for quantitative determination of donepezil in human plasma. 150 μL of plasma samples were placed in 2.2 mL 96-deepwell plates and both donepezil and loratadine (IS) were extracted from human plasma by liquid–liquid extraction (LLE), using hexane as the organic solvent. Robotic liquid handling workstations were employed for all liquid transfer and solution preparation steps and resulted in a short sample preparation time. After vortexing, centrifugation and freezing, the supernatant organic solvent was evaporated and reconstituted in a small volume of reconstitution solution. The method developed, includes a sample analysis performed by reversed phase LC–MS/MS, with positive ion electrospray ionization, using multiple reaction monitoring (MRM). The chromatographic run time was set for 2.0 min with a flow rate of 0.7 mL/min in a C_{18} analytical column. The method was significantly sensitive, specific, accurate and precise for the determination of donepezil in human plasma and had the shortest run time. The curve was proven to be linear for the concentration range of 0.1–100 ng/mL. After validation, the method was applied to the rapid and reliable quantitative determination of donepezil in a bioequivalence study after per os administration of a 5 mg donepezil tablet.

Keywords: Donepezil; Bioequivalence; 96-Well format; LC–MS/MS

1. Introduction

Donepezil hydrochloride [(±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimethoxyindan-1-one hydrochloride] is a selective, reversible acetylcholinesterase inhibitor, acting in the central nervous system (CNS). It is the second drug approved by the FDA for the treatment of Alzheimer's disease. Current theories on the pathogenesis of the cognitive signs and symptoms of Alzheimer's disease attribute some of them to a deficiency of cholinergic neurotransmission. Donepezil hydrochloride is postulated to exert its therapeutic effect by enhancing cholinergic function. This is accomplished by increasing the concentration of acetylcholine through reversible inhibition of its hydrolysis by acetylcholinesterase [1–4].

Analytical methods so far reported, employ HPLC coupled with ultra violet detector [5–11], fluorescence detector [12,13] and single mass spectrometry detector [14]. Only one tandem mass spectrometric method [15] has been reported for the determination of donepezil enantiomers, as well as one method employing capillary electrophoresis coupled with ultra violet detector [16]. None of the methods mentioned above is automated, while analysis run time in all cases exceeds 15 min.

The objective of the present work was to develop and validate the first automated, high throughput, 96-well format based LLE, LC–MS/MS method for the determination of donepezil in human plasma. Robotic liquid handling systems were employed to working solutions and plasma standards preparation, to all liquid transfer steps included in the sample preparation procedure as well as to the addition/removal of the organic solvent. The current method includes a simple and rapid sample preparation, as a result of the robotic systems and the 96-well format plates utilization that enabled parallel processing, as well as a significantly

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shorter chromatographic run time compared to those appearing in previous publications [5–16]. After successful development and validation, it was applied to the reliable high-throughput determination of donepezil in a bioequivalence study after per os administration of a 5 mg tablet in 24 healthy volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Donepezil hydrochloride was supplied by Rafarm Pharmaceutical Company (Athens, Greece) and loratadine used as the IS was purchased from Sigma–Aldrich (Athens, Greece). Acetonitrile, methanol, *n*-hexane 97% (HPLC grade), ammonium acetate, glacial acetic acid and ammonium hydroxide (analysis grade) were also purchased from Sigma–Aldrich. All aqueous solutions and buffers were prepared using de-ionized and doubly distilled water (resistivity > 18 MΩ) from a Millipore Milli-Q Plus System (Malva, Athens, Greece). Pooled human control plasma (heparinized) was kindly donated from Ippokrateio Hospital (Athens, Greece).

2.2. Instrumentation and software

A Perkin-Elmer Multiprobe II HT-EX workstation (Perkin-Elmer, Downers Grove, IL, USA) equipped with an 8-tip robotic arm and controlled by WinPrep Software (Version 1.17.0216) was employed for all liquid transfer steps. Two hundred microlitres conductive disposable tip-boxes were purchased from E&K Scientific Products (Cambell, CA, USA) while a tipchute, reagent troughs and a tip flush/wash station were purchased from Perkin-Elmer. Two microlitres eppendorf microfuge tubes (Lab Supplies, Athens, Greece), 2.2 mL square 96-deepwell plates and eppendorf deepwell mats for covering the 96-well plates (Sigma–Aldrich), were also used in sample preparation procedure. A Tomtec Quadra 96 model 320 robotic liquid handling system equipped with a 96-tip pipetting head (Bidservice, NJ, USA) was employed for organic solvent addition as well as supernatant organic layer transferring after extraction into a new 2.2 mL 96-deepwell plate. An Eppendorf 5810 R (Bacakos, Athens, Greece) centrifuge compatible with both 96-well plates and Eppendorf microfuge tubes was also utilized during sample preparation. Evaporation was performed into a Zymark TurboVap 96-well format plate evaporator (Malva) by the application of nitrogen gas flow, produced by an Agilent Nitrogen Generator (Duratec, Hockenheim, Germany), connected with a SF4 Air Compressor (Atlas Copco, Athens, Greece). A 96-well plate vortex-mixer (MS1 Minishaker) was bought from Metrolab (Athens, Greece). The HPLC system consisted of an Agilent 1100 series binary pump, a degasser and a column oven/cooler (Hellamco). The fact that six 96-deepwell plates could be placed inside the CTC PAL autosampler (Hellamco) allowed the automated analysis of a large number of samples. Finally, the HPLC system was coupled to a PE Sciex API 3000 triple quadrupole mass spectrometer (Biosolutions, Athens, Greece) via a turbo ionspray source. The mass spectrometer operated under the Analyst 1.4.1 software, which was utilized for all data acquisition. All pharmacokinetic parameters were estimated by using Pharsight WinNonLin 5.0.1 statistical software.

2.3. Chromatographic conditions

Chromatography was carried out at ambient temperature, on a YMC Pack ODS-A analytical column, 5 μm, 200 Å (50 mm × 4.0 mm i.d.) (Schermbeck, Germany). HPLC elution mobile phase consisted of 82% acetonitrile and 18% 10 mM ammonium acetate, adjusted to pH 5.0 (v/v) with glacial acetic acid, at a flow rate of 0.7 mL/min. The injection volume was 20 μl and the total run time was set for 2.0 min. The autosampler temperature was set at 10 °C while the system pressure during the analysis was about 340–360 psi.

2.4. Mass spectrometric conditions

Analyte and IS were detected by monitoring the precursor → product ion transition using multiple reaction monitoring (MRM) scan mode. The MRM was performed at *m/z* 380.6 → 91.2 for donepezil and 383.3 → 337.2 for the IS (Fig. 1). The turbo ionspray of the mass spectrometer was operated in the positive ionization mode while tuning parameters were optimized for donepezil and loratadine (IS) by infusing a 100 ng/mL standard solution containing both compounds in mobile phase at 20 μL/min via an external syringe pump (Harvard 11 plus) directly connected to the mass spectrometer. The turbo ionspray source temperature was maintained at 420 °C and the turbo ionspray voltage was set at 1500 V. Declustering potential (DP) was set at 71 V for donepezil and 46 V for the IS while curtain gas was set at 8 (arbitrary units). The nebulizer gas (GS_1) was set at 14 (arbitrary units) while the turbo ionspray gas (GS_2) at 7 L/min. The collision-induced dissociation (CID) gas was set at 6 (arbitrary units) and the collision energy was set at 65 and 33 V for donepezil and IS, respectively.

2.5. Preparation of standard and quality control/method validation samples

Master standard solutions of donepezil {100 μg/mL (SD_1)} and loratadine {100 μg/mL (IS_1)} were prepared by dissolving each of the accurately weighed reference compounds in MeOH. Solution IS_2 (50 ng/mL in MeOH/H_2O 1:1, v/v) was daily prepared by diluting IS_1 solution in order to be used in sample preparation procedure. Working solutions of 2000, 1000, 400, 200, 100, 40, 20, 10, 4 and 2 ng/mL for donepezil were prepared by serial dilutions of SD_1 with MeOH/water 50/50 (v/v). A separate weighing of donepezil was performed so as to prepare a Quality Control/Method Validation (QC/MV) master standard solution (100 μg/mL, SD_1'). Serial dilutions of SD_1' were also performed to prepare four levels of QC working solutions, 1500, 150, 6 and 2 ng/mL. All working solutions were prepared in 2 mL eppendorf tubes and were stored along with the master standard solutions at 4 °C.

The calibration curve consisted of a blank sample (matrix sample processed without IS), a zero sample (matrix sample

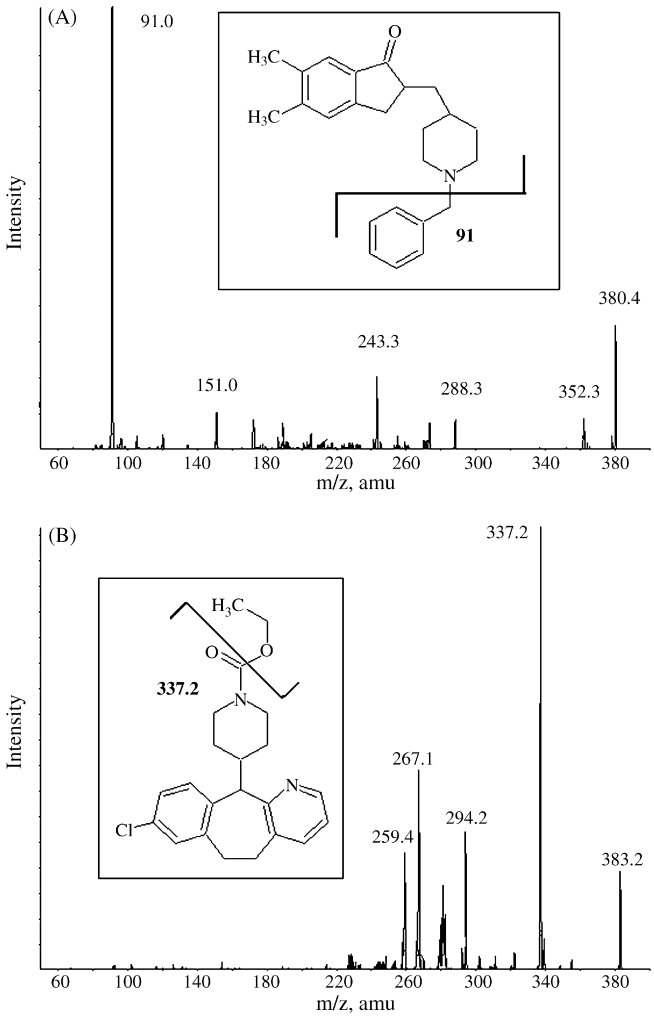

Fig. 1. Product ion spectra of donepezil (A) and IS (B) generated by collision induced dissociation of the corresponding parent ions.

processed with IS) and 10 non-zero standards covering the expected range of concentrations to be quantified. Calibration standards, QC and MV samples were prepared in the same biological matrix (human plasma) as the samples to be analyzed. Working solutions were diluted 20 times with human plasma so as to obtain final standard concentrations of 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 ng/mL. The limit of quantitation (LOQ) was defined as the lowest calibration standard concentration and should have a S/N ratio > 10 in all curves. QC/MV samples concentrations were as follows: MV_L (0.1 ng/mL), MV_1/QC_1 (0.3 ng/mL), MV_2/QC_2 (7.5 ng/mL) and MV_3/QC_3 (75 ng/mL). All standards were to be prepared in bulk, dispensed in 2.0 mL aliquots in eppendorf tubes and finally stored at −30 °C.

2.6. Sample extraction and preparation

Stored plasma samples were thawed at room temperature, vortexed and centrifuged at 3500 rpm for 5 min at approximately 4 °C. The eppendorf tubes were decapped and placed into 24-position microfuge racks on the deck of the Multiprobe along with 2.2 mL 96-deepwell plates and two troughs containing the reagents to be added (IS_2 solution and ammonium hydroxide 10%). Multiprobe transferred 150 μL of each of the calibration, quality control and subject samples from the eppendorf tubes into the appropriate wells of a 96-well plate employing 200 μL disposable conductive tips. Then, to each well, 50 μL of IS_2 solution and 75 μL of ammonium hydroxide 10% were added. The 96-well plates were removed from the Multiprobe and after vortex mixing for 10 min they were placed on the Tomtec where 1300 μL of hexane were added into all 96 wells of each plate. Plates were covered with a mat and analyte and IS were extracted by vortex mixing for 20 min. Then, the plates were centrifuged for 15 min, at 3500 rpm and 4 °C and frozen for 1 h at −30 °C. 900 μL of the supernatant organic layer were transferred into new 2.2 mL 96-deepwell plates. The plates were then placed into the Zymark TurboVap 96-well format plate evaporator, and the organic extracts were evaporated to dryness with a nitrogen flow at 60 °C. Dry residues were reconstituted by the addition of 250 μL of mobile phase, plates were vortex mixed for 5 min and finally placed into the autosampler for direct injection.

2.7. Method validation

The developed analytical method was fully validated, according to US Food and Drug Administration (FDA) bioanalytical method validation guidance [17]. The method calibration curve contained 10 non-zero standards ranging from 0.1 to 100 ng/mL, a range that was suitable for a pharmacokinetic study after per os administration of a 5 or 10 mg tablet of donepezil. QC and MV samples had two distinct purposes: the results for QC samples provided the basis for accepting or rejecting analytical runs, while the results for MV samples were used to calculate the bias and precision of the assay methodology. Accuracy and intra-, inter-run precision were assessed during the method validation by analyzing MV samples, as defined above, in five runs on separate days. The %accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values.

Extraction recovery of the analytical method was assessed by comparing analyte peak area counts from plasma samples fortified with analyte at three concentration levels (0.3, 7.5 and 75 ng/mL) as well as IS at 50 ng/mL prior to extraction, to plasma samples fortified with analyte and IS at the same concentrations post-extraction. Recovery was determined by using data from all five runs. Samples stability was assessed while inside the autosampler as well as after four freeze and thaw cycles compared to fresh ones for two concentration levels (low–medium S_l and medium–high S_h). Moreover, aliquots of S_l and S_h samples were prepared and maintained at room temperature for 6 h, period which exceeds the usual time that samples remain at room temperature before analysis, so as for short-term stability to be assessed. To evaluate long-term stability, aliquots of the two sample-types were initially frozen at −20 °C for 60 days, thawed and analyzed. Finally, stock and working solutions stability (stored at 4 °C) was estimated by comparing fresh and old dilutions in mobile phase.

2.8. Matrix effect study

Matrix effect was determined by comparing analyte peak area counts from plasma samples fortified with analyte at three concentration levels (0.3, 7.5 and 75 ng/mL) as well as IS at 50 ng/mL post extraction, to samples from neat solutions at the same concentrations for analyte and IS. Numerical values (%) for each concentration level were calculated by dividing the area of plasma extracted sample spiked with analyte and IS, by the area of the respective neat solution. Matrix effect profiles for the whole chromatographic run were investigated by the application of the post-column infusion protocol [18,19]. Blank plasma extracts were injected in the LC–MS/MS system by the simultaneous post-column infusion of a mixture of the analyte and the IS at 100 ng/mL in mobile phase via the Harvard syringe pump. The flow rate was set at 50 μL/min while the syringe pump was connected in parallel with the ionization source via a PEEK tee.

3. Results and discussion

The current method allows the automated, high-throughput donepezil monitoring in human plasma by the use of automated LLE and LC–MS/MS. General laboratory experience has proven LLE to provide cleaner extracts than the other extraction techniques, as evidenced by fewer matrix effects and less tendency for backpressure build-up in the chromatographic column, as more samples are injected [20]. A 96-well format based on deep well plates was used, as a result of the low organic volume (1300 μL) involved in LLE. This format, along with the use of the robotic liquid handling systems Multiprobe and Tomtec, resulted in a shorter sample preparation time, reducing at the same time the possibility of human error as time-consuming and labor-intensive manual pipetting steps were bypassed. All liquid transfer steps, such as working and plasma standard solutions preparation, reagents addition and organic solvent transfer were automatically performed. Moreover, the use of 96-well format allowed the simultaneous evaporation of 192 samples (two 96-well plates).

As far as the chromatographic profile of the method is concerned, the retention times were about 0.98 min for donepezil and 1.27 min for IS with a total run time of 2.0 min (Fig. 2) when the shortest run time so far reported for donepezil analysis was 15 min [10]. Chromatographic parameters were optimized by testing several mobile phase compositions including MeOH and ACN as the organic part of the mobile phase and formic acid, formate and acetate buffer as the aqueous part adjusted in pH 5.0, 4.0 or 3.5. Best results were obtained by the use of ACN along with ammonium acetate buffer pH 5.0. Organic/aqueous mobile phase ratio was tested for several values with the final composition of 82% of ACN along with 18% of ammonium acetate buffer 10 mM pH 5.0 being the mobile phase composition of choice, as it provided the best chromatographic result. Finally, a short (50 mm) C_{18} chromatographic column was chosen so as to achieve a short chromatographic run time for analysis. The latter along with the use of the autosampler allowed the analysis of several hundreds of samples overnight, making possible

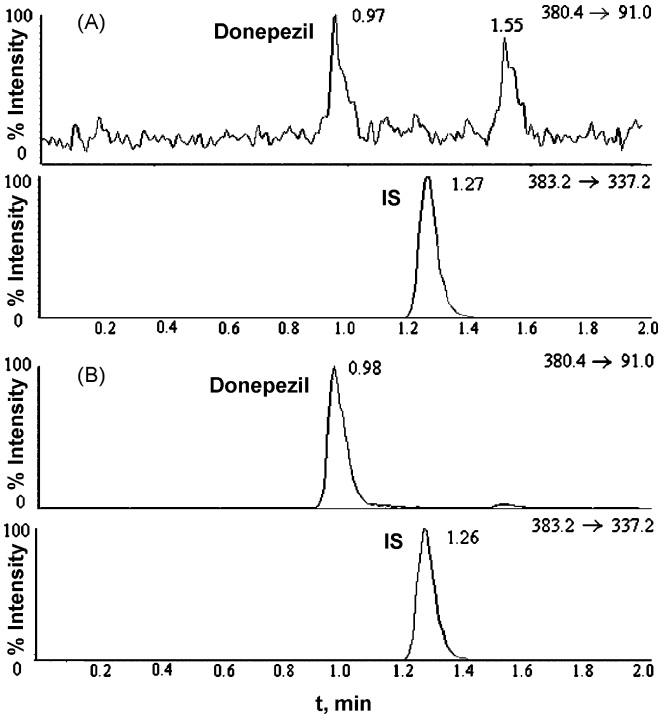

Fig. 2. Representative MRM chromatogram of donepezil (top) and IS obtained from MV_L (A) and MV_2 (B) sample during pre-study validation.

the completion of multi-sample studies, such as bioequivalence studies, within only a few days.

The method was proven to be highly sensitive with a Limit of Quantification (LOQ) of 0.1 ng/mL for donepezil. Absence of chromatographic interferences was certified by the analysis of a zero and a blank plasma samples. The mean regression coefficient (*R*-squared) for the five runs was 0.999, average linear slope 0.989 was ($S_a = 0.004$) and average intercept was 0.095 ($S_b = 0.150$). The experimental values of *F*-test (Mandel) were smaller than 2.903, when the (theoretical) threshold value of *F*-distribution (5%, one-sided) was 5.590. Consequently, the calibration curves were linear in the specific range. The result of the proportionality test was also positive; the *t*-test experimental value of 0.635 was greatly smaller than the theoretical value of 2.306 (5%, two-sided). As a result, the concentration data produced by the 96-well LLE procedure applied in this method was satisfactory for donepezil standard samples.

As far as accuracy and precision are concerned, all values were within the acceptable range. Data for accuracy and both intra- and inter-run precision (expressed as CV%) are presented in Table 1. The extraction method presented sufficient recovery of the analyte and the IS from plasma. Loratadine was selected as an IS, because it has almost the same molecular weight and the same number of rings (4) with donepezil. Therefore, it was expected to show a pretty similar behavior during LLE procedure. Extraction recovery values for both molecules were practically the same, so one can assume that this method can be expanded for other matrices, too. The specific extraction procedure was chosen among several alternatives tried in method development. More specifically, sample pH was adjusted by using NaOH and NH_4OH solutions, with the latter providing

Table 1
Intra- and inter-assay accuracy and precision results

MV sample	%Intra-run accuracy[a]	%Inter-run accuracy[b]	Intra-run precision[c] (%CV)	Inter-run precision[b] (%CV)
MV_L (0.1 ng/mL)	100.0	98.4	13	10
MV_1 (0.3 ng/mL)	104.2	100.6	8.9	7.7
MV_2 (7.5 ng/mL)	104.8	107.5	8.3	2.3
MV_3 (75 ng/mL)	108.1	103.7	8.0	5.0

[a] $n = 6$, expressed as 100 × (mean calculated concentration)/(nominal concentration).
[b] Values obtained from all five runs ($n = 30$).
[c] $n = 6$.

Table 2
Extraction recovery results (mean values ± S.D.)

Mean area values ($n = 5$)	Sample code	Sample conc. (ng/mL)	Extraction recovery (%)	Matrix suppression (%)
Donepezil	Low	0.3	52.7 ± 4.2	68.5 ± 5.6
	Medium	7.5	58.8 ± 2.8	73.7 ± 4.3
	High	75	58.0 ± 3.6	92.8 ± 2.1
IS	–	50	59.5 ± 3.1	75.5 ± 3.8

significantly better results. As far as LLE organic solvent is concerned, several options were examined including methyl *t*-butyl ether, ethyl acetate, *n*-hexane and isopropyl alcohol as well as mixtures of the above. Most sufficient and repeatable extraction was achieved by the use of *n*-hexane as the extraction solvent. Extraction recovery as well as matrix effect results are presented in Table 2. The post-column infusion matrix effect study proved weak ion suppression to be taking place at the retention time of donepezil and the IS (Fig. 3). Finally, the results of all stability tests (data not shown) were within the acceptable range.

3.1. Application to a bioequivalence study

A bioequivalence study of donepezil comparing a test formulation (produced by RAFARM Pharmaceutical Company) versus a reference formulation (Aricept®/Pfizer), was conducted according to the approved protocol, the ethical principles that have origins in the Declaration of Helsinki and the Good Laboratory Practice (GLP) regulatory requirements. The study design was an open single-dose, two-treatment, two-period crossover with a washout period of 21 days between the two periods and were in compliance with the EMEA (European Agency for the Evaluation of Medicinal Products) guidelines, subsection CPMP/EWP/QWP/1401/98, on the investigation of bioavailability and bioequivalence (London, 26 July 2001).

An equal number of subjects (24 healthy volunteers from the local population plus 4 substitute volunteers) were randomly assigned to each of the two possible dosing sequences. After the administration of a single dose of each formulation (5 mg/tab), under fasting conditions, blood samples (14 per subject per period) were collected at designated times (0–192 h) and analyzed with the current method (Fig. 4). The pharmacokinetic parameters that were analyzed are $AUC_{0–192}$, $AUC_{0–\infty}$, C_{max}, T_{max}, k_{el} and $T_{1/2}$. The primary parameters were $AUC_{0–192}$ and C_{max} while the secondary parameters were $AUC_{0–\infty}$, T_{max}, k_{el} and $T_{1/2}$. For donepezil, analysis of variation was performed on Ln-transformed data for $AUC_{0–192}$, $AUC_{0–\infty}$, and C_{max}. A non-parametric analysis was carried on T_{max}. Ratios of least square means and 90% geometric confidence intervals were calculated for Ln-transformed data for $AUC_{0–192}$, $AUC_{0–\infty}$ and

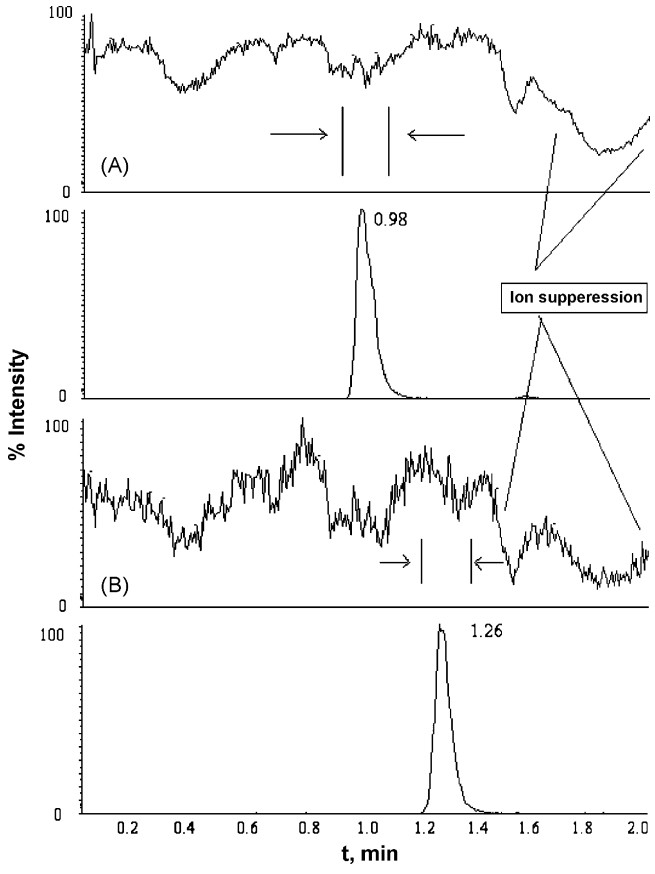

Fig. 3. Study of the matrix effect phenomenon by means of continuous infusion of donepezil (A) and IS (B) at 100 ng/mL and parallel injection of extracts of blank plasma samples.

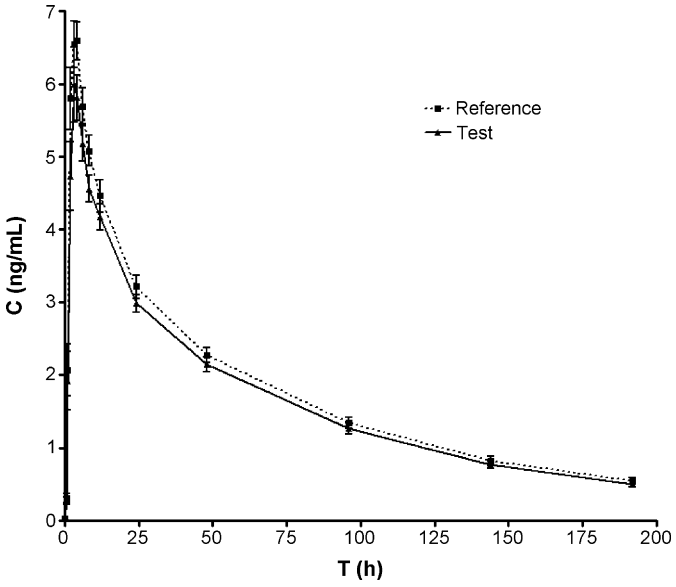

Fig. 4. Mean plasma concentration–time curves from 24 subjects after per os administration of each of the two donepezil tablet formulations (5 mg).

Table 3
Pharmacokinetic parameters (mean values ± S.D.) of donepezil after oral administration of a single dose of a 5 mg tablet of each formulation in 24 volunteers

Parameter	Values
$AUC_{0–192\,h}$ (ng h/mL)	
Test	318.6 ± 67.3
Reference	342.3 ± 77.7
$AUC_{0–\infty}$ (ng h/mL)	
Test	372.2 ± 87.1
Reference	399.8 ± 103.7
C_{max} (ng/mL)	
Test	6.50 ± 2.23
Reference	7.12 ± 1.59
T_{max} (h)	
Test	3.67 ± 1.40
Reference	3.38 ± 1.14
Test vs. reference (90% confidence intervals)	
$AUC_{0–192\,h}$	[89.21, 97.51]
$AUC_{0–\infty}$	[89.62, 97.72]
C_{max}	[83.14, 95.83]

C_{max}. Intra- and inter-subject coefficient of variations were also calculated. Bioequivalence was to be concluded in the 90% geometric confidence intervals of the ratio of the means (T/R) for the Ln-transformed data for $AUC_{0–192}$, $AUC_{0–\infty}$ and C_{max}. In the current study concerning donepezil, these were fully included within 80–125%. Mean pharmacokinetic parameters obtained from concentration data are presented in Table 3.

4. Conclusions

A novel, automated 96-well format LLE, LC–MS/MS method for the quantitative determination of donepezil in human plasma has been presented. The method was proven to be highly sensitive (LOQ, 0.1 ng/mL) and specific because of the employment of a tandem mass spectrometric detector. The utilization of two liquid-handling robotic systems minimized the time of sample preparation and the possibility of human error as well as simplifying the overall procedure. Contrary to other methods so far reported, the chromatographic run time of the analysis was significantly short (2.0 min). All factors mentioned above, allowed the successful application of the validated method to a bioequivalence study.

Acknowledgement

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