Research article

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A rapid and sensitive LC-MS/MS method for quantification of donepezil and its active metabolite, 6-*o*-desmethyl donepezil in human plasma and its pharmacokinetic application

Nageswara Rao Pilli,[a] Jaswanth Kumar Inamadugu,[b] Neeraja Kondreddy,[b] Vijaya Kumari Karra,[a] Rajasekhar Damaramadugu[b] and J. V. L. N Seshagiri Rao[c]*

ABSTRACT: A rapid and sensitive liquid chromatography–tandem mass spectrometric (LC-MS/MS) assay method has been developed and fully validated for simultaneous quantification of donepezil and its active metabolite, 6-*o*-desmethyl donepezil in human plasma. Analytes and the internal standard were extracted from human plasma by liquid–liquid extraction technique using a 30:70 v/v mixture of ethyl acetate and *n*-hexane. The reconstituted samples were chromatographed on a C_{18} column by using a 70:30 v/v mixture of acetonitrile and ammonium formate (5 mM, pH 5.0) as the mobile phase at a flow rate of 0.6 mL/min. The calibration curve obtained was linear ($r \geq 0.99$) over the concentration range of 0.09–24.2 ng/mL for donepezil and 0.03–8.13 ng/mL for 6-*o*-desmethyl donepezil. The results of the intra-day and inter-day precision and accuracy studies were well within the acceptable limits. The proposed method was successfully applied for the estimation of the drug in real time plasma samples for pharmacokinetic studies. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: donepezil; 6-*o*-desmethyl donepezil; quantification; human plasma; LC-MS/MS

Introduction

Alzheimer's disease is a progressive neurodegenerative disease associated with a deficit of neurotransmitter acetylcholine levels in the brain (Davies and Maloney, 1976). Donepezil (Fig 1) [2-(1-Benzyl-piperidin-3-ylmethyl)-5, 6-dimethoxy-indan-1-one] is a reversible centrally and selectively acting acetylcholinesterase inhibitor (Sugimoto *et al*., 1995; Kosasa *et al*., 2000). It is a well tolerated drug that enhances acetylcholine levels of the brain. The drug undergoes first-pass metabolism in the liver by the hepatic enzymes CYP 3A4 and CYP 2D6 (Tiseo *et al*., 1998; Rogers and Friedhoff, 1998). 6-*o*-Desmethyl donepezil is one of the major metabolites of donepezil, possessing a pharmacological activity similar to donepezil. Donepezil was the second drug approved by the US Food and Drug Administration (FDA), in 1996, for the treatment of Alzheimer's disease.

For the determination of donepezil in human plasma some high-performance liquid chromatographic methods (Haginaka and Seyama, 1992; Lee *et al*., 1992; Matsui *et al*., 1999a; Yasui-Furukori *et al*., 2002; Radwan *et al*., 2006; Nakashima *et al*., 2006) and liquid chromatography–mass spectrometric methods (Matsui *et al*., 1995; Matsui *et al*., 1999b; Hao *et al*., 2003; Lu *et al*., 2004; Xie *et al*., 2006; Apostolou *et al*., 2007; Patel *et al*., 2008; Shah *et al*., 2009) have been reported. Most of the LC-MS/MS methods reported so far were only for quantification of donepezil alone. Of all the above, only two methods are comparable with the present work. The method proposed by Patel *et al*. (2008) for quantification of donepezil and 6-*o*-desmethyl donepezil in human plasma utilizes solid-phase extraction and the run time is greater than 5 min. Another method reported by Matsui *et al*. (1999b) for determination of donepezil enantiomers in human plasma is more sensitive but complicated.

In the present investigation, we have developed a method having a shorter run time with simple liquid–liquid extraction

* Correspondence to: J. V. L. N. Seshagiri Rao, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530 003, India. E-mail: jvlnsrao@rediffmail.com

[a] University College of Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad-500 085, India

[b] Analytical and Environmental Chemistry Division, Department of Chemistry, Sri Venkateswara University, Tirupati-517 502, India

[c] College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530 003, India

Abbreviations used: DPZ, donepezil; DDPZ, 6-*o*-desmethyl donepezil; FDA, US Food and Drug Administration.

technique. The following are the advantages of the proposed method over those reported earlier: (1) because of the use of less plasma volume (300 μL), the volume of the sample to be collected per time point from an individual during the study is reduced significantly—this allows inclusion of additional points; (2) employing a single-step liquid–liquid extraction procedure minimizes the chances of errors, saves considerable time and simplifies the sample preparation procedure; (3) greater sensitivity is achieved even with low plasma volumes and the method is well suited for pharmacokinetic analysis; and (4) the rapid sample turnaround time of 2.7 min makes it an attractive procedure in high-throughput bioanalysis of donepezil and its active metabolite, 6-*o*-desmethyl donepezil in human plasma.

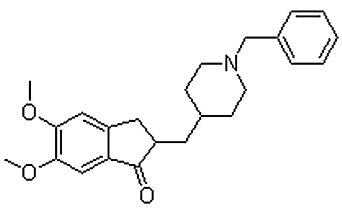
Experimental

Chemicals and reagents

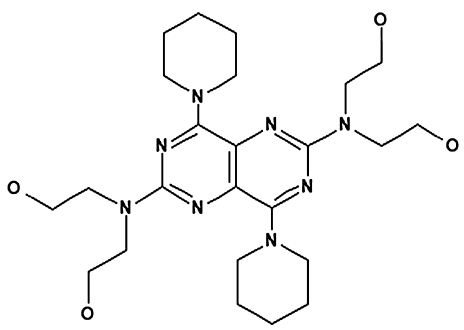
The reference sample of donepezil (DPZ) (>99.81%) and 6-*o*-desmethyl donepezil (DDPZ) (>96.44%) were purchased form Neucon Pharma Pvt. Ltd, Goa, India and dipyridamole (Fig 1) (>99.80%), used as an internal standard in this study, was obtained from Dr Reddy's Laboratories Ltd, Hyderabad, India. Water used for the LC-MS/MS analysis was prepared using a Milli Q water purification system procured from Millipore (Bangalore, India). Acetonitrile and methanol were of HPLC grade and purchased from J. T. Baker (Phillipsburg, USA). Analytical-grade ammonium formate and formic acid were purchased from Qualigens (Glaxo Mumbai, India). The control human plasma sample was procured from Cauvery Diagnostics and Blood Bank (Secunderabad, India).

Instrumentation and chromatographic conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Chromolith® speedROD, an RP C_{18} column (50 × 4.6 mm, 5 μm; Waters Corporation, Ireland), a binary LC-20AD prominence pump, an autosampler (SIL-HTc) and a solvent degasser (DGU-$20A_3$) was used for the study. Aliquots of the processed samples (20 μL) were injected into the column, which was kept at room temperature. The isocratic mobile phase, a 70:30 v/v mixture of acetonitrile and ammonium formate (5 mM, pH 5.0) was delivered at 0.6 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MS-MS detection in positive ion mode for both the analytes and the internal standard using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray™ interface at 500°C. The ion spray voltage was set at 5000 V. The source parameters viz. the nebulizer gas, curtain gas, auxillary gas and collision gas were set at 35, 10, 40 and 4 psi, respectively. The compound parameters viz. the declustering potential, collision energy, entrance potential and collision cell exit potential were 86, 35, 10 and 6 V for DPZ, 86, 68, 10 and 6 V for DDPZ, and 130, 62, 10 and 18 V for IS. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 380.2 precursor ion to the *m/z* 91.1 for DPZ, *m/z* 366.2 precursor ion to the *m/z* 91.2 for DDPZ and *m/z* 505.4 precursor ion to the *m/z* 385.2 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data obtained were processed using Analyst software™ (version 1.4.2).



Donepezil (DPZ)



Dipyridamole (IS)

Figure 1. Chemical structures of donepezil (DPZ) and dipyridamole (IS).

Standard solutions

Primary stock solutions of DPZ and DDPZ for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions (0.5 mg/mL of DPZ and 0.2 mg/mL of DDPZ) of the analytes were prepared in methanol and stored at 2–8°C; they were found to be stable for one month (data not shown). From these stock solutions, appropriate dilutions were made using a 50:50 v/v mixture of methanol and water as a diluent to produce two-in-one working standard solutions of 483, 362, 243, 121, 48.6, 24.3, 12.1, 3.64 and 1.82, and 163, 122, 81.7, 40.8, 16.3, 8.17, 4.08, 1.22 and 0.61 ng/mL of DPZ and DDPZ respectively. These solutions were used to prepare the relevant calibration curves (CC) standards. Another set of working solutions of DPZ and DDPZ (two-in-one) was prepared in the diluent (from primary stock) at concentrations of 387, 240, 62, 6 and 1.88, and 135, 84, 28, 2 and 0.66 ng/mL, respectively, to be used as QC samples. The primary stock solution of dipyridamole (1.0 mg/mL) was prepared in 0.1 M HCl in methanol. A working concentration of the internal standard (100 ng/mL of dipyridamole) solution was prepared in the diluent. These working solutions were stored at 2–8°C for 30 days.

The calibration curve and quality control samples were prepared by spiking 50 μL of the working solution (containing both DPZ and DDPZ) into 950 μL of control plasma. Calibration samples for DPZ and DDPZ were made at concentrations of 24.2, 18.1, 12.1, 6.07, 2.43, 1.21, 0.61, 0.18 and 0.09, and 8.13, 6.09, 4.08, 2.04, 0.82, 0.41, 0.20, 0.06 and 0.03 ng/mL, respectively. Quality control samples for DPZ and DDPZ were prepared at concentrations of 20.6, 6.96 (higher quality control, HQC), 14.4, 4.87 (middle quality control 2, MQC2), 3.46, 1.17 (middle quality control 1, MQC1), 0.28, 0.09 (lower quality control, LQC) and 0.09, 0.03 (lower limit quality control, LLOQ QC) ng/mL.

Sample processing

A 300 μL volume of the plasma sample was transferred to a 15 mL glass test tube, and into it 50 μL of working concentration of the IS (100 ng/mL) was spiked. After vortexing for 30 s, a 4 mL aliquot of the extraction solvent (ethyl acetate–*n*-hexane, 30:70 v/v) was added using Dispensette Organic (Brand GmbH, Wertheim, Germany). The sample was shaken for 10 min using a reciprocating shaker (Scigenics Biotech, Chennai, India) and then centrifuged for 4 min at 4000 rpm using a Heraeus Megafuse 3SR, Japan centrifuge. The organic layer (3.0 mL) was transferred to a 5 mL glass test tube and evaporated at 45°C under a stream of nitrogen. The dried extract was reconstituted with 200 μL of the mobile phase and a 20 μL aliquot was injected into the column.

Method validation

A thorough validation of the method was carried out as per the US FDA guidelines (FDA, 2001). The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. Selectivity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of the DPZ and DDPZ are acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. The responses of the interfering substances or background noise at the retention time of the internal standard are acceptable if they are less than 5% of the mean response of internal standard in LLOQ samples.

Sensitivity was established from the background noise or response from six spiked LLOQ samples. The six replicates should have a precision of ≤20% and an accuracy of ±20%. Matrix effect was investigated to ensure that precision, selectivity and sensitivity were not compromised by the matrix. Matrix effect was checked with six different lots of EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total).

Linearity was tested for DPZ and DDPZ in the concentration range of 0.09–24.0 ng/mL and 0.03–8.13 ng/mL, respectively. For the determination of linearity, standard calibration curves containing at least nine points (non-zero standards) were plotted and checked. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. The acceptance limit of accuracy for each of the back-calculated concentrations was ±15% except for LLOQ, where it was ±20%. For a calibration run to be accepted, at least 75% of the standards, including the LLOQ and ULOQ, were required to meet the acceptance criterion, otherwise the calibration curve was rejected. Five replicate analyses were performed on each calibration standard. The samples were run in the order from low to high concentration.

Intra-assay precision and accuracy were determined by analyzing six replicates at five different QC levels on two different days. Inter-assay precision and accuracy were determined by analyzing six replicates at five different QC levels on five different runs. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it should be ±20% and a precision of ≤15% relative standard deviation (RSD), except for LLOQ QC, where it should be ≤20%.

Recovery of the analytes from the extraction procedure was determined by comparing the peak areas of the analytes in spiked plasma samples (six each of low, medium2 and high QCs) with the those of the analytes in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analytes at the step immediately

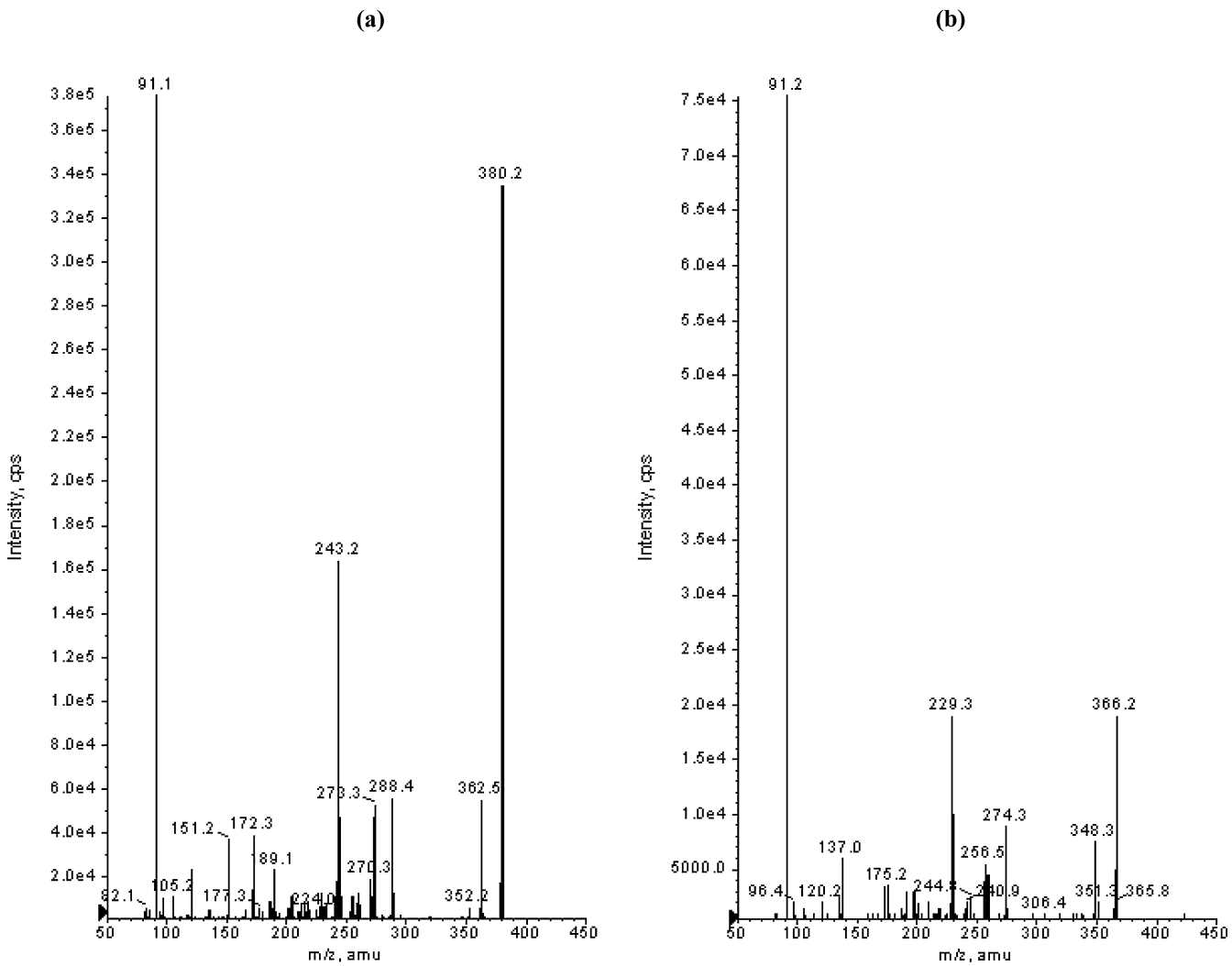


Figure 2. (a) Product ion mass spectra of $[M + H]^+$ of DPZ. (b) Product ion mass spectra of $[M + H]^+$ of DDPZ. (c) Product ion mass spectra of $[M + H]^+$ of IS.

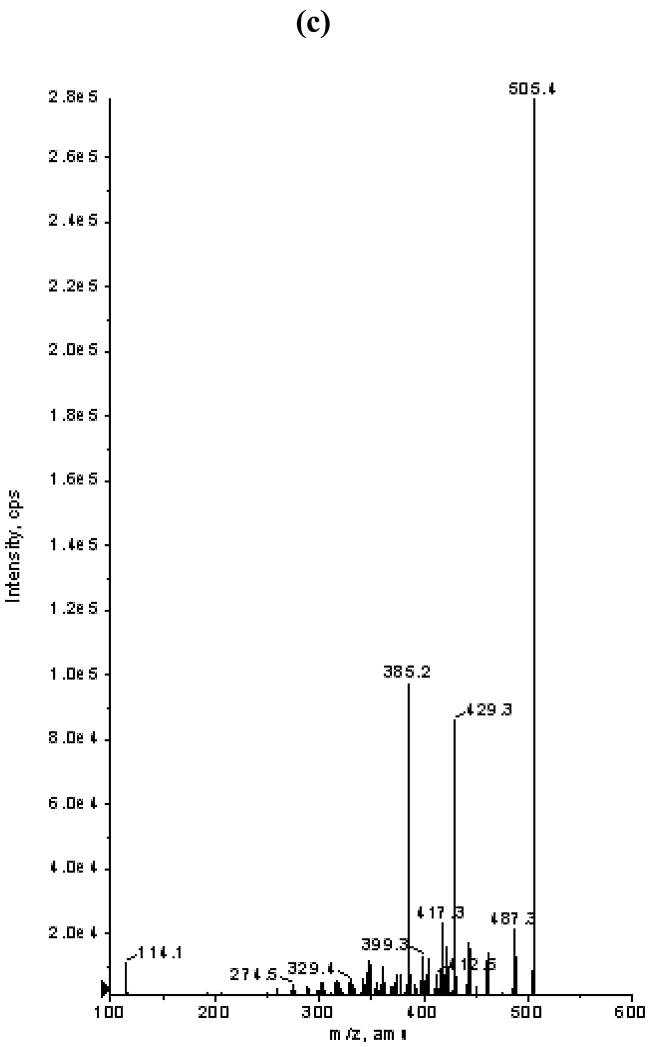


Figure 2. *Continued.*

prior to chromatography. Similarly, recovery of the IS was determined by comparing the mean peak areas of the extracted QC samples ($n = 6$) with those of the IS in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The dilution integrity exercise was performed with the aim of validating the dilution test to be carried out on higher analyte concentrations above the ULOQ during real-time analysis of subject samples. Dilution integrity experiment was carried out at 1.7 times the ULOQ concentration for both the analytes. Six replicates each of half and quarter concentrations were prepared and their concentrations were calculated by applying the dilution factors 2 and 4.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8°C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability (7 h), processed sample stability (autosampler stability for 48 h, wet extract stability for 24 h and reinjection stability for 24 h), freeze–thaw stability (three cycles) and long-term stability (60 days) were tested at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15%) and precision (≤15% RSD).

Pharmacokinetic study design

A pharmacokinetic study on the drug was performed in healthy male subjects ($n = 6$). Blood samples were collected following oral administration of 10 mg tablet of Donepezil at pre-dose and 0.5, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.25, 3.75, 4, 4.25, 4.5, 4.75, 5, 6, 8, 10, 12, 16, 24, 48, 144, 240 and 360 h, in EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at −70°C until use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle 1, middle 2 and high concentration levels were assayed in triplicate and were distributed among the unknown samples in the analytical run; not more than 33% of the QC samples were greater than ±15% of the nominal concentration. The plasma concentration–time profile of each analyte was analyzed by non-compartmental method using WinNonlin Version 5.1.

Results and discussion

Method development

Mass parameters were tuned in both positive and negative ionization modes for the analytes. Good response was found in positive ionization mode. Data of the MRM mode were considered to obtain better selectivity. The product ion mass spectra of DPZ and DDPZ and IS were presented in the Figs 2a, 2b, 2c.

Separation was attempted using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C_8 and C_{18} of different makes like Chromolith, Hypersil, Hypurity Advance, Zorbax, Kromasil and Intersil. Use of a buffer with the desired pH (5.0) helped in achieving good response for MS detection in positive ionization mode. A mobile phase consisting of acetonitrile and 5 mM ammonium formate (70:30 v/v; pH 5.00 ± 0.05) was found to be suitable, as the analytes were protonated and well separated in this phase. A Chromolith® speedROD, RP_{18e}, 50 × 4.6 mm, HL 5 µm column gave a good peak shape and response even at LLOQ level for both analytes and IS. The mobile phase was operated at a flow rate of 0.6 mL/min. The retention times of DPZ, DDPZ and IS were low enough (1.6, 1.5 and 1.6 min), allowing a small run time of 2.7 min.

Liquid–liquid extraction (LLE) technique was employed for the sample preparation in this work. LLE is helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system, and also minimizing the experimental cost. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS. Among the different solvents checked alone and in combination for their suitability, ethyl acetate in combination with *n*-hexane in the ratio of 30:70, v/v was found to be optimal, which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analytes from the plasma.

A good internal standard must mimic the analyte during extraction and compensate for any analyte on the column. For LC-MS/MS analysis, the use of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. Isotope-labeled analyte was not available to serve as IS, so in the initial stages of this work several compounds were investigated to find a suitable IS. Dipyridamole was found to be best for the present purpose. Extraction recovery of the internal standard was almost the same as that of the analytes.

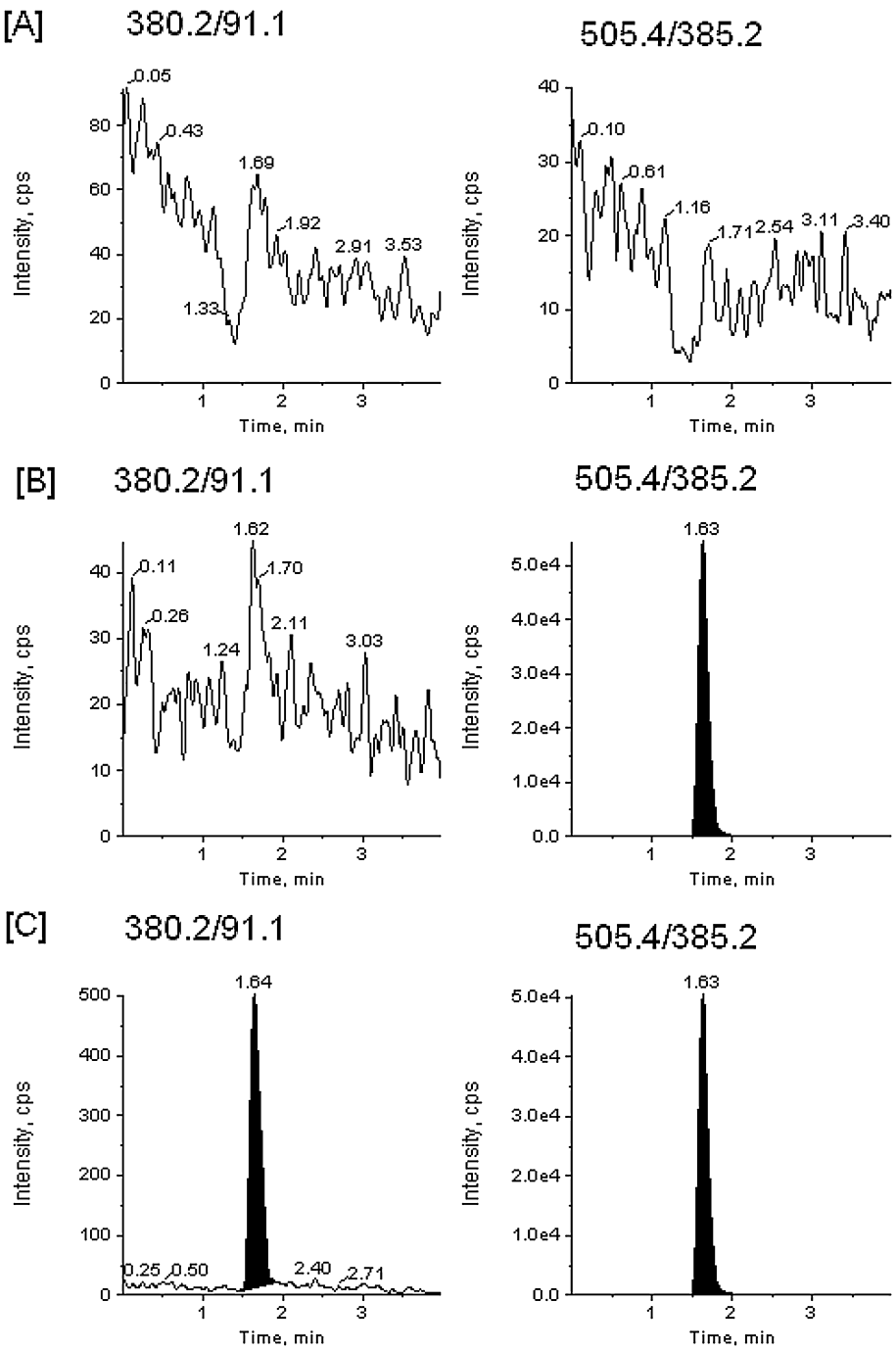


Figure 3. Typical MRM chromatograms of DPZ (left panel) and IS (right panel) in (A) human blank plasma; (B) human plasma spiked with IS; and (C) a LLOQ sample along with IS.

Selectivity and chromatography

The degree of interference by endogenous plasma constituents with the analytes and IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Figs 3 and 4, no significant direct interference in the blank plasma traces were observed from endogenous substances in drug-free plasma at the retention time of the analytes.

Sensitivity

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 2.72 and 102%, and 4.48 and 103% for DPZ and DDPZ, respectively.

Matrix effect

No significant matrix effect was observed in all the six batches of human plasma for the analytes at LQC and HQC concentrations. The precision and accuracy for DPZ at LQC concentration were found to be 5.77 and 96.5%, and at the HQC level were 1.44 and 92.3%, respectively. Similarly, the precision and accuracy for

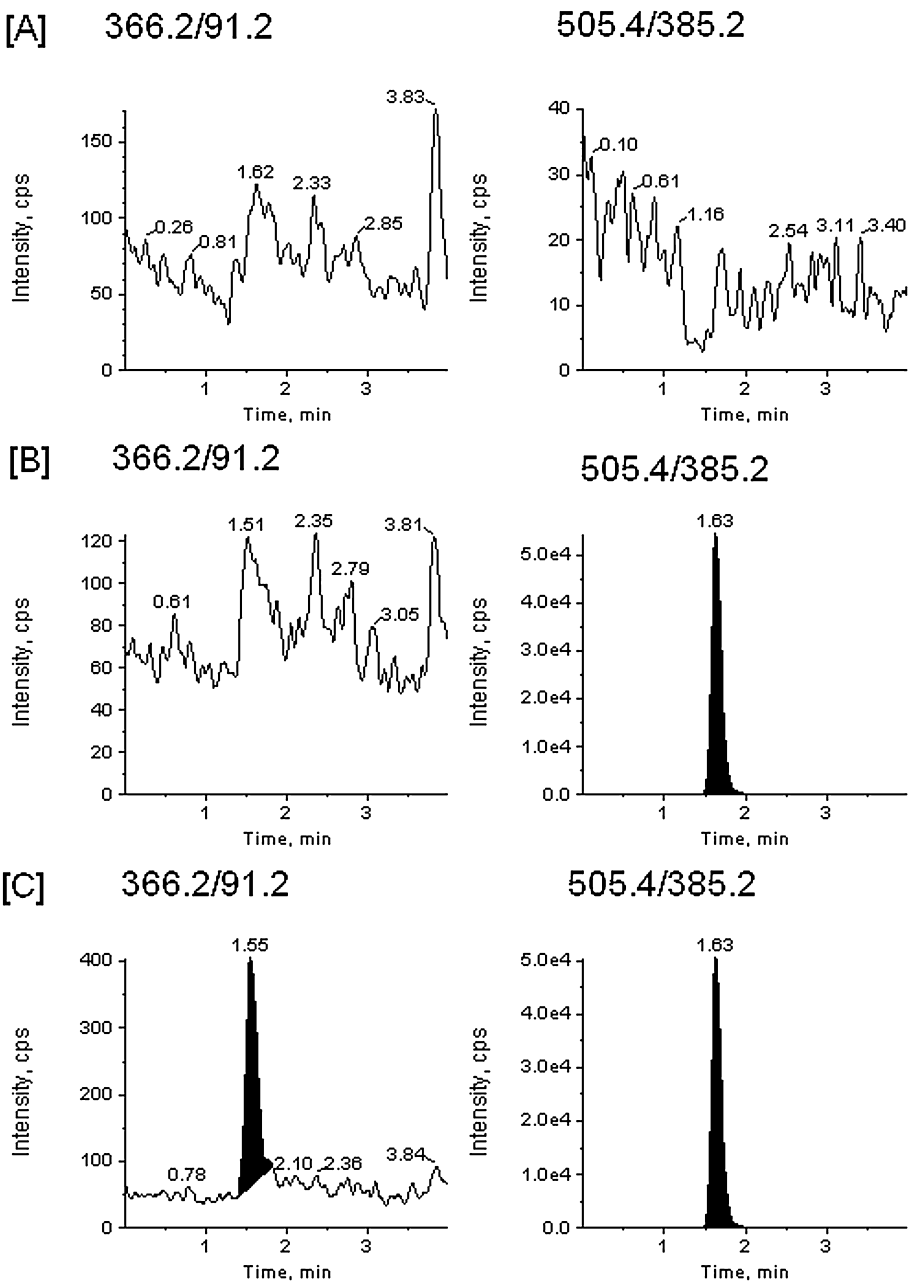


Figure 4. Typical MRM chromatograms of DDPZ (left panel) and IS (right panel) in (A) human blank plasma; (B) human plasma spiked with IS; and (C) a LLOQ sample along with IS.

DDPZ at LQC concentration were found to be 2.03 and 92.2%, and at HQC level were 1.09 and 91.1% respectively.

Linearity

Nine-point calibration curve was found to be linear over the concentration range of 0.09–24.2 ng/mL for DPZ and 0.03–8.13 ng/mL for DDPZ. After comparing the two weighting models ($1/x$ and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship for both the analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99.

Precision and accuracy

As shown in Table 1, the precision and accuracy of each analyte in the intra-day and inter-day runs were within ±15% at LQC, MQC1, MQC2 and HQC concentrations and within ±20% at LLOQ QCs.

Extraction efficiency

Six replicates at low, medium and high quality control concentration for DPZ and DDPZ were prepared for recovery determination. The recovery comparison samples of IS were compared against the response of IS in the middle QC level. The mean recovery was 77.9, 78.5 and 75.5% for DPZ, DDPZ and IS respectively.

Table 1. Precision and accuracy of the method for determining donepezil and *o*-desmethyl donepezil in plasma samples

Analytes	Concentration added (ng/mL)	Intra-day precision and accuracy (n = 12; 6 from each batch) Concentration found (mean; ng/mL)	Precision (%)	Accuracy (%)	Inter-day precision and accuracy (n = 30; 6 from each batch) Concentration found (mean; ng/mL)	Precision (%)	Accuracy (%)
Donepezil	0.09	0.09	7.32	105	0.09	9.36	97.8
	0.28	0.28	8.18	103	0.27	8.17	97.1
	3.46	3.31	2.35	95.6	3.26	4.51	94.3
	14.4	13.6	2.57	94.4	13.4	5.32	92.9
	20.6	19.2	1.88	93.1	19.1	3.31	92.9
o-Desmethyl donepezil	0.03	0.03	9.67	103	0.03	9.83	98.7
	0.09	0.08	8.95	90.4	0.09	8.01	95.1
	1.17	1.11	4.31	94.6	1.09	4.13	93.4
	4.87	4.58	1.98	94.0	4.55	4.27	93.3
	6.96	6.32	2.70	90.9	6.49	3.58	93.2

Table 2. Stability samples result for donepezil and *o*-desmethyl donepezil (n = 6)

Analytes	Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/stability (%)	Precision (%)
Donepezil	Process[a]	0.28	0.26 ± 0.01	93.2	3.30
		20.6	19.5 ± 0.82	94.7	4.20
	Process[b]	0.28	0.29 ± 0.01	103	3.02
		20.6	20.0 ± 0.32	97.1	1.60
	Bench top[c]	0.28	0.31 ± 0.01	111	4.40
		20.6	19.2 ± 0.23	93.1	1.19
	FT[d]	0.28	0.25 ± 0.01	91.3	2.09
		20.6	19.3 ± 0.61	93.5	3.17
	Reinjection[e]	0.25	0.27 ± 0.02	108	5.64
		18.7	20.3 ± 1.42	109	7.00
	Long-term[f]	0.28	0.27 ± 0.02	101	6.76
		20.6	20.7 ± 1.65	107	7.98
o-Desmethyl donepezil	Process[a]	0.09	0.09 ± 0.00	97.9	4.90
		6.96	6.79 ± 0.23	97.6	3.33
	Process[b]	0.09	0.09 ± 0.01	96.5	8.20
		6.96	6.89 ± 0.25	99.1	3.61
	Bench top[c]	0.09	0.09 ± 0.01	93.9	7.06
		6.96	6.45 ± 0.12	92.6	1.81
	FT[d]	0.09	0.01 ± 0.00	103	3.05
		6.96	6.21 ± 0.31	89.1	5.06
	Reinjection[e]	0.09	0.09 ± 0.01	108	8.37
		6.50	6.98 ± 0.47	107	6.71
	Long-term[f]	0.09	0.09 ± 0.00	96.1	3.70
		6.96	6.97 ± 0.09	104	1.34

[a] After 48 h in autosampler at 10°C; [b] after 24 h in refrigerator at 2–8°C; [c] after 7 h at room temperature; [d] after three freeze and thaw cycles; [e] after 24 h of reinjection; [f] at −70°C for 60 days.

Dilution integrity

The upper concentration limits could be extended to 41.1 ng/mL for DPZ and 13.8 ng/mL for DDPZ by half and quarter dilutions with screened human blank plasma. The mean back-calculated concentrations for half and quarter dilution samples were within 85–115% of their nominal value. The coefficients of variation (%CV) for half and quarter dilution samples were less than 10%.

Stability

In the different stability experiments carried out, viz. bench top stability (7 h), autosampler stability (48 h), repeated freeze–thaw cycles (three cycles), reinjection stability (24 h), wet extract stability (24 h at 2–8°C) and long-term stability at −70°C for 60 days, the mean percentage nominal values of the analytes were found to be within ±15% of the predicted concentrations for the

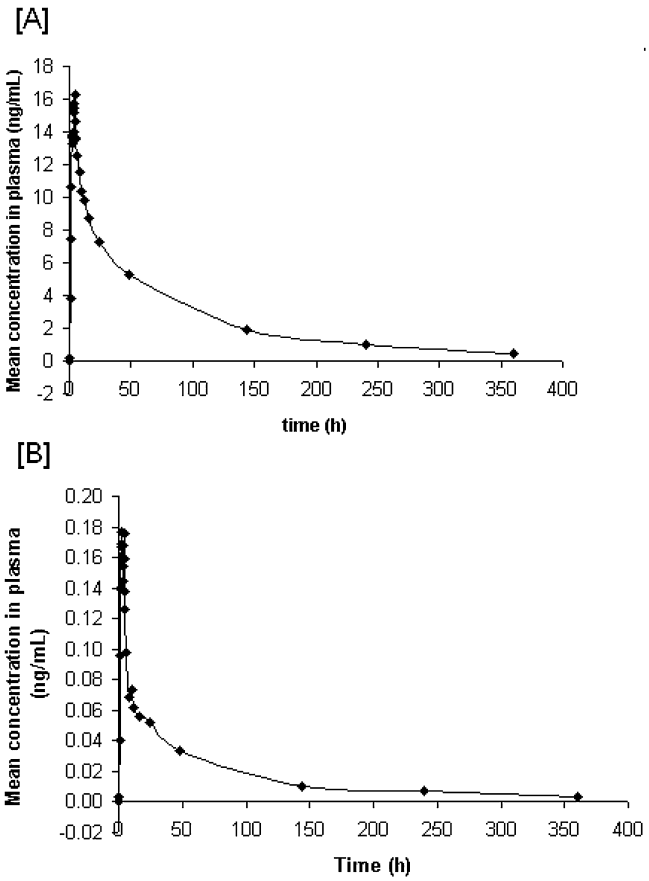


Figure 5. Mean plasma concentration–time profile of (A) DPZ (B) DDPZ in human plasma following oral dosing of DPZ tablet to healthy volunteers.

analytes at their LQC and HQC levels (Table 2). Thus, the results were found to be within the acceptable limits during the entire validation.

Application to the real human plasma samples

In order to verify the sensitivity and selectivity of this method in a real-time situation, the present method was used to test for donepezil and 6-*o*-desmethyl donepezil in human plasma samples collected from healthy male volunteers ($n = 6$). The mean plasma concentrations vs time profiles of DPZ and DDPZ are shown in Fig. 5. The maximum concentration in plasma (C_{max}), time point of C_{max} (T_{max}), half-life ($t_{1/2}$), area under the plasma concentration–time curve from zero hour to the last measurable concentration (AUC_{0-t}) and area under the plasma concentration–time curve from zero hour to infinity ($AUC_{0-\infty}$) for DPZ were 17.1 ± 2.08 ng/mL, 3.70 ± 1.01 h, 106 ± 23.9 h, 949 ± 191 ng.h/mL and 1023 ± 222 ng.h/mL, respectively and for DDPZ was 0.22 ± 0.05 ng/mL, 2.60 ± 0.91 h, 68.0 ± 35.5 h, 5.91 ± 2.70 ng.h/mL and 6.78 ± 2.66 ng.h/mL, respectively.

Conclusion

The LC-MS/MS assay presented in this paper is rapid, simple, specific and sensitive for quantification of donepezil and 6-*o*-desmethyl donepezil in human plasma and is fully validated according to commonly acceptable FDA guidelines. The method showed suitability for pharmacokinetic studies in humans. The extraction method gave consistent and reproducible recoveries for the analytes and IS from plasma. The cost-effectiveness, simplicity of the assay, use of liquid–liquid extraction and sample turnover rate of less than 2.7 min per sample made it possible to analyze more than 400 plasma samples per day.

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