## **Research Article**

## Development and validation of an ultra-high performance liquid chromatography photodiode array method for the quantification of donepezil in human plasma and its application

Rasda Boonprasert\*, Tippanate Keawvijit, Supawadee Pakdeenukoolkijja

Clinical Toxicology Laboratory, Siriraj Poison Control Center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

## ABSTRACT

An ultra-high performance liquid chromatography-photodiode array detection (UHPLC-PDA) method was developed and validated for determination of the concentration of donepezil in patient's plasma. Plasma spiked with diphenhydramine as an internal standard was used for the solid phase extraction. The eluent solution was diluted with 0.05% trifluoroacetic acid and injected into an UHPLC system. Chromatographic separation was performed on a reverse phase column (1.8  $\mu$ m, 100 mm x 2.1 mm I.D.) and using acetonitrile with 0.05% trifluoroacetic acid in milli-Q water as the mobile phase. The gradient program for the mobile phase involved a flow rate of 0.45 mL/min and 3 min total run time. The photodiode array (PDA) detector was chosen to operate at 230 nm. The retention times were 1.70 and 2.11 min for donepezil and diphenhydramine, respectively. The method was developed and fully validated according to United Stated Food and Drug Administration (USFDA) guidance. The linearity of the method revealed a coefficient of determination or square of r greater than 0.998 in the concentration range 10-250 ng/mL. Extraction recoveries ranged from 84.6-85.6% with good repeatability. A simple, rapid, and reproducible UHPLC/PDA method for quantifying the concentration of donepezil in patient's plasma was thus developed and completely validated. This method was successfully utilized to measure the plasma concentration of 105 Thai patients with Alzheimer's disease and vascular dementia.

#### **Keywords**:

Donepezil, UHPLC-PDA, Therapeutic drug monitoring

#### **1. INTRODUCTION**

Donepezil (DP) is an acetylcholinesterase inhibitor (AchE-I) which can reduce the degradation of acetylcholine and has been authorized for the treatment of mild to moderate Alzheimer disorder (AD)<sup>1</sup>. It has been shown that there is a statistically significant correlation between increased plasma concentrations of donepezil in plasma and acetylcholinesterase inhibition; whereby at a done-pezil plasma concentration of 15.6 ng/ml, 50% of acetylcholinesterase activity was inhibited, while above 50 ng/ml, 80-90% of inhibition was achieved<sup>2</sup>. A higher oral dosage was thus recommended in that study for an improved therapeutic response toward the mentioned

outcomes. However, therapeutic response of donepezil is usually about 20-30%. Inter-individual variability may additionally occur from genetic factors in particular polymorphisms of gene, which is a necessary function in many pharmacokinetic systems<sup>3</sup>. Therefore, therapeutic drug monitoring (TDM) could also be a beneficial device to predict the medical results of donepezil in the treatment of Alzheimer's disease<sup>4-5</sup>.

Several studies have suggested the use of HPLC with UV for quantitation of donepezil in pharmacokinetic studies, pharmaceutical preparations and TDM<sup>6-8</sup>. However while the most common method have high sensitivity, their limitation include long chromatographic run times and the need for an additional extraction

\*Corresponding author:

<sup>\*</sup>Rasda Boonprasert Email: rasda.boo@mahidol.ac.th



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process. The highest sensitivity can be achieved by using liquid chromatography-tandem mass spectrometry (LC-MS/MS)<sup>9-13</sup>, but this is a difficult technique to apply. Consequently, there is a need for a high sensitivity, short chromatographic run time, simple technique to fulfill the requirements for the use in therapeutic drug monitoring. In this present study, a sensitive, selective and fast ultra high performance liquid chromatography photodiode array method (UHPLC-PDA) method was developed for determination of donepezil in patient's plasma. The method was validated in line with Guidance for Industry, Bio-analytical Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (USFDA CDER, 2018, BP)<sup>14</sup>. This method can be utilized for the determination of donepezil plasma concentration on TDM studies.

#### 2. MATERIALS AND METHODS

#### 2.1. Instruments and analytical conditions

An Acquity<sup>TM</sup> Ultra Performance Liquid Chromatography (Waters Corporation, Milford, MA, USA) used to be the separation module. Chromatographic separation was performed on an ACQUITY UPLC® BEH HSS T3 column (1.8 µm, 100 mm x 2.1 mm I.D.) (Waters Corporation, Milford, MA, USA). To obtain the appropriate result, the mobile phase was carried out with a flow rate of 0.45 mL/min and a column temperature of 40°C. The gradient program for the mobile phase was optimized using acetonitrile and 0.05% trifluoroacetic acid (TFA) in milli-Q water. An ACQUITY UPLC<sup>®</sup> Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA) was operated at wavelengths between 200-380 nm and quantitation was optimized on the requirement for high sensitivity and specificity. The auto-sampler was conditioned at 8°C and the injection volume was 10 µL. Empower 2 software program (Waters Corporation, Milford, MA, USA) was used for data management.

#### 2.2. Chemicals and reagents

Donepezil hydrochloride and diphenhydramine (internal standard, IS) were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany) and their chemical structure are presented in Figure 1. HPLC grade acetonitrile and methanol were purchased from Labscan Ltd. (Bangkok, Thailand). The water used for the experiments was made through milli-Q<sup>®</sup> water purification system (EMD Millipore, Billerica, MA, USA). All other chemicals used were of analytical grade. Drug-free human plasma was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital.



**Figure 1.** Chemical structures of donepezil (A) and diphenhydramine (B).

# **2.3. Standard solution preparation and calibration curve**

The donepezil hydrochloride and diphenhydramine standard were accurately weighed. Primary standard stock solutions of each were prepared in dimethyl sulfoxide (DMSO) with a final concentration of 50,000 ng/mL. Working standard solutions were prepared by diluting of the stock standard solutions with a mixture of methanol and water (50:50, v/v) to a final concentration that varied between 500-12,500 ng/mL to be used as calibration standards (CSs). Six different concentrations of CS were organized by spiking the working standard solutions in drug-free human plasma to give the desired concentration; a 10, 50, 100, 150, 200 and 250 ng/mL. Meanwhile, the quality control samples (OC) were prepared separately in the same way to create low, medium, and high concentration controls at 30, 120 and 220 ng/mL, respectively. The IS working standard solution was prepared by diluting the primary standard stock solutions with a combination of methanol and water (50:50, v/v) to a final concentration of 200 ng/mL. All standard stock solutions, working solutions, and QC samples were stored at -20°C untill use

#### 2.4. Sample preparation

For solid phase extraction (SPE), OASIS HLB: Hydrophilic-Lipophilic-Balanced reversed- phase sorbent 30 mg 1 mL (Waters Corporation, Milford, MA, USA) was used for sample preparation. SPE HLB cartridges were first conditioned with 1 mL of methanol and equilibrated with 1 mL of milli-Q water before use. Next, 20 µL of IS (200 ng/mL) was added into 1.0 mL of the plasma samples in 2.0 mL micro-centrifuge tube, and 200 µL orthophosphoric acid was then added and mixed immediately. Then, 1 mL aliquots of the plasma samples were aspirated into the wetted preconditioned SPE HLB cartridges. The plasma components were then washed in two steps with wash solvent, 1 mL ammonia solution in water (2:98, v/v) and then 1 mL of ammonia solution and 20% methanol in water (2:98, v/v). Donepezil were then eluted from the dried columns using 0.5 mL acetic acid in methanol (2:98, v/v). Next, 200 µL of the eluent was then diluted with 200 µL of 0.05% trifluoroacetic acid before being injected into the UHPLC system.

## 2.5. Method validation

The developed method was fully validated according to the Guidance for Industry, Bio-analytical Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (USFDA CDER, 2018, BP)<sup>14</sup>. The method's selectivity was examined using six sources of drug-free plasma, for which the ideal result should not have interfering peaks at the retention times of DP and IS. The sensitivity at the lower limit of quantification (LLOQ) was also examined through dilution of the DP standard in plasma, which was extracted and then quantified at the lowest detectable concentration with signal to noise ratio (S/N) greater than 5.

The linearity and calibration curve were represented by the means of a linear regression model, y=mx+b, where y was the ratio of peak area of DP to the peak area of IS, and x is the concentration at different levels, here comprising 10, 50, 100, 150, 200 and 250 ng/mL. All the calibration levels yielded linear relationships with coefficient of determination ( $r^2$ ) that exceeded 0.995.

Stability studies for the auto-sampler, benchtop, freeze-thaw, stock solution, and long-term stability aspects were performed using three replicates of QC) samples at the lower (LQC), middle (MQC), and higher (HQC). The stability of each specific condition was calculated by examination the samples amount under the same conditions to that of the freshly prepared samples. The accuracy and precision were acceptable when within  $\pm 15\%$ , while the extraction efficiency should be more than 80% and the coeffocoent of variance (%CV) must be within  $\pm 15\%$ .

## **3. RESULTS AND DISSCUSION**

## 3.1. Method development

The mobile phase compositions were optimized using a mixture of the standard solution of DP and IS. The isocratic program was optimized with the initial ratio of acetonitrile and 0.05% TFA in milli-Q water varying in the range of 30-50%. The aim was to determine the constituents and separate the co-eluting peaks. The isocratic condition could achieve the seperation with acetonitrile and 0.05% TFA in milli-Q water at a ratio of 30:70, v/v, but this method has a long analytic time with peak tailing. Therefore, to get the most suitable results, the gradients of mobile phase were optimized. It was found that by performing the separation via 32:68, v/v at 0 min, 35:65, v/v at 1-2.5 min and 32:68, v/v at 3 min, DP and IS can be separated as well. The retention factor (k') was 1.83 for DP and 2.51 for IS, while the resolution factor was 4.69. Although, the retention factor was lower than 2, this method demonstrated an excellent separation of DP from the void peak, as shown in Figure 2. The ACQUITY UPLC<sup>®</sup> Photodiode Array (PDA) Detector from Waters Co. Ltd. (USA) was used at wavelengths between 200-380 nm. The PDA spectra in Figure 2 shown that the best possible sensitivities of DP and IS was 229.8 nm and 220.0 nm, respectively. However, the specific wavelength for this method was chosen at 230 nm. Here, the wavelength selection was



Figure 2. The PDA spectra of donepezil (DP) and diphenhydramine (DPH).

based totally on the requirement for achieving the highest sensitivity and specificity for DP determination.

#### 3.2. Method development: sample preparation

This optimized protocol was able to further removes interferences so a cleaner extract is achieved. A wash-elute study was performed to determine the optimal retention and elution parameter for given drugs on the Oasis sorbent depending on the organic concentration and pH. In reverse phase Oasis<sup>®</sup> HLB SPE, the retention of a drug is influenced by the organic concentration and pH. Donepezil is a basic drug with a tertiary amine group (pKa=8.82). Basic drugs will be more retained at a high pH value (unionized) and less retained at a lower pH value (ionized). Thus, ammonia solution in water and 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% methanol MeOH in water (2:98, v/v) were determined to give the maximum % MeOH for the wash solution. Then, acetic acid in water and 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% MeOH in water were determined to give the minimum % MeOH for the eluent solution. Finally, ammonia solution in water (2:98, v/v) and ammonia solution in 20% MeOH in water (2:98, v/v) were chosen for the wash solution while acetic acid in in MeOH (2:98, v/v) was chosen for the eluent solution.

#### 3.3. Assay performance and validation

#### 3.2.1. Selectivity and sensitivity

Six sources of drug-free plasma were studied for assessing the selectivity. There were no interfering peaks at the retention time of DP and IS in selectivity testing. The representative UPLC-PDA chromatogram acquired are presented in Figure 3 for the internal standard sample (A) and the clinical plasma sample (B). The



Figure 3. Representative UHPLC-PDA chromatograms obtained from the (A) internal standard sample and (B) clinical plasma sample after an oral administration of 10 mg donepezil.

lower limit of quantification (LLOQ) was 10.0 ng/mL with 10.33 signals to noise ratio. Thus, the developed method was shown to be selective and sensitivity for DP and IS.

#### 3.2.2. Linearity and calibration standard curve

The six-point calibration standard curve was found to be linear over the concentration range of 10-250 ng/mL. The calibration standard curve was developed by plotting DP to IS peak area ratio (y) towards the DP concentration (x), This method also confirmed to give excellent linearity, with a coefficient of determination ( $r^2$ ) greater than 0.998 (0.9985, 0.9983, 0.9992 for three consecutive days) as shown in Figure 4, which was within the acceptance limits and in accordance with the USFDA guidance. The percentage accuracy (or percentage relative error, %RE) and percentage precision (or percentage coefficient of precision, %CV) were within  $\pm 20\%$  at LLOQ and  $\pm 15\%$  at other concentrations. The results are summarized in Table 1.

## 3.2.3. Extraction efficiency

Assessment of the extraction efficiency of the method was carried out based on 3 replicates analyses of the LQC, MQC, and HQC by evaluating the peak areas of the extracted samples with the peak areas of non-extracted standard solutions at the same concentrations.

The mean absolute recoveries of the LQC, MQC and HQC have been 85.1%, 84.6% and 85.6%, respectively, for DP and 75.0% for IS. The results indicated that the sample preparation of this method was consistent, precise and reproducible with %CV equal to 4.18, 3.50, 3.24 and 3.01 for LQC, MQC, HQC and IS, respectively.

## 3.2.4. Accuracy and precision

The within-run and between-run accuracy and precision were examined through the analysis of six replicates of the LLOQ, LQC, MQC, and HQC for three consecutive days. The within-run and between run accuracies of the method were in the range of 91.38 to 106.47 % RE and 97.99 to 103.03 % RE, respectively. Within-run and between run precision was additionally observed, with % CV in the range of 1.22 to 3.90% and 1.59 to 3.69%, respectively. The results are summarized in Table 2. These results indicated that the developed method had sufficient accuracy and precision to determine DP in human plasma.

## 3.2.5. Stability study

Stability of the analysis was assessed by testing three replicates of plasma spiked with three QC concentrations under various conditions and comparing the result with freshly prepared samples. DP was proved to be stable in human plasma under the specified storage



Figure 4. Calibration standard curve of donepezil (day 3) constructed by plotting the peak-area ratios of the donepezil plasma concentration compared to the IS against nominal concentration.

Table 1. Precision (%CV) and accuracy (%RE) of donepezil calibrations standards curve.

Nominal concentration	Measured concentration <sup>a</sup>	Precision	Accuracy	
(ng/mL)	Mean±SD (ng/mL)	(%CV)	(%RE)	
10.00	10.14±0.79	7.78	101.41	
50.00	49.28±3.21	6.15	98.55	
100.00	99.16±4.00	4.03	99.16	
150.00	152.61±2.67	1.75	101.74	
200.00	199.53±2.61	1.31	99.76	
250.00	249.29±3.86	1.55	99.72	

<sup>a</sup>Mean, n=3 sets of calibration curve

Nominal concentration	Day	Measured value	Accuracy (%RE)		Precision (%CV)	
(ng/mL)		Mean±SD (ng/mL)	Within run <sup>b</sup>	Between run <sup>c</sup>	Within run <sup>b</sup>	Between run <sup>c</sup>
10.00	1	10.00±0.62	100.02	99.71	6.17	4.28
	2	10.06±0.33	100.58	99.71	3.32	4.28
	3	9.85±0.31	98.52	99.71	3.19	4.28
30.00	1	29.95±1.08	99.83	100.03	3.61	2.99
	2	30.07±0.73	100.24	100.03	2.41	2.99
	3	30.013±0.96	100.03	100.03	3.19	2.99
120.00	1	117.97±2.78	98.31	97.99	2.36	3.69
	2	121.08±1.95	100.90	97.99	1.61	3.69
	3	113.70±4.43	94.75	97.99	3.90	3.69
220.00	1	224.30±2.74	101.95	101.60	1.22	1.59
	2	225.445±2.96	102.48	101.60	1.31	1.59
	3	220.78±3.51	100.36	101.60	1.59	1.59

<sup>b</sup> Six replicates (n=6) at each concentration.

<sup>c</sup> Three day runs (n=18) at each concentration.

 Table 3. Stability of donepezil (n=3).

Stability	Nominal concentration (ng/mL)	Precision (%CV)	Accuracy (%RE)	% differences
Auto-sampler stability	30.00	1.05	101.47	2.38
(10 hrs., 8°C, n=3)	120.00	1.22	99.63	2.33
	220.00	0.83	101.42	1.36
Bench-top stability	30.00	1.33	99.42	0.31
(4 hrs., 25°C, n=3)	120.00	1.13	102.69	5.48
	220.00	3.63	102.81	2.75
Freeze and thaw stability	30.00	3.47	100.17	1.07
(3 cycles, n=3)	120.00	2.74	102.08	4.85
	220.00	1.49	107.36	7.29
Long term stability	30.00	1.22	103.08	4.00
(90 days, -20°C, n=3)	120.00	2.70	97.93	0.59
	220.00	0.73	100.44	0.38

n=number of replicates

conditions regarding the auto-sampler stability (10 h. in the auto-sampler at 8°C), bench-top stability (4 h. at room temperature 25°C), freeze-thaw stability (three cycles) and long-term stability (90 days at -20°C). The percentage differences in the accuracy (%RE) and precision (%CV) were within the acceptable range of  $\pm 15\%$ . The results are summarized in Table 3. These results indicated that DP was stable in plasma throughout the sample preparation procedure and under the storage conditions.

## 3.2.6. Application

The method was efficiently used to quantify the steady state donepezil plasma concentration in Thai

patients with Alzheimer's disease and vascular dementia. The measured drug concentration of 105 patient's plasma after the administration of donepezil at 10 mg maintenance dose was 36.31-137.31 ng/mL<sup>15</sup>. The study was approved by the Siriraj Institutional Review Board (539/2559(EC1)) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

#### 4. CONCLUSIONS

An UHPLC-PDA method was developed and proved to be simple, rapid, sensitive and reproducible method for the determination of donepezil concentrations in the patient plasma. It was thoroughly validated and found to perform within acceptable limit in accordance with the USFDA standard guideline. Moreover, this method was efficiently utilized to measure the plasma concentration of Thai patients with Alzheimer's disease and vascular dementia.

## 5. ACKNOWLEDGEMENT

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#### **Conflict of interest**

The authors declare that they have no conflict of interest.

#### Funding

None to declare.

## **Ethics approval**

This study and was approved by the Siriraj Institutional Review Board (SIRB) No. 539/2559(EC1).

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