

3. Analytical and Bioanalytical Methods

3.1 Introduction

Analytical methods play a crucial role in formulation design, discovery and manufacturing of pharmaceuticals. They are essential to quantify drug molecules at all stages of pharmaceutical product development like drug design, synthesis, formulation development, stability studies, estimation of *in vitro* and *in vivo* efficacy of the molecules. Thus, it is essential to develop an accurate and precise analytical method that is simple, easy in sample preparation, accurate, reliable and reproducible. Moreover, the method should be specific and sensitive to assess the quality, safety and performance attributes in a simple and cost-effective manner [1,2]. The developed methods may show variation in the results due to changes in instrument, analyst and laboratories. Validation of the developed method plays a vital role in minimizing these method related problems. Guidelines for developing and validating analytical or bio-analytical methods were laid down by regulatory bodies like US-Food and Drug Administration (US-FDA), International Conference of Harmonization (ICH) and European Medicines Agency (EMA) lay emphasis on the method suitability and in compliance with the standards set for specificity, sensitivity, accuracy, precision and recovery [3–5].

3.2 Analytical methods for estimation of donepezil

Donepezil is official in the USP40-NF35 and the monograph describes the quantification by spectroscopy and chromatography methods. Few analytical and bio-analytical methods have been reported earlier for the quantification of donepezil in bulk, formulation and biological matrices. Most of the reported methods are having its own drawbacks related to sensitivity, utilization of higher portions of organic solvents and for biological samples require large sample volume with a complex sample preparation procedure [6–9].

Particularly for biological samples few methods have also been reported by liquid chromatography tandem mass spectroscopy (LC-MS/MS) [10–12]. However, usage of advanced technologies such as LC-MS/MS is very expensive and that may hamper routine analysis for various applications.

For the present study, quantitative analysis of donepezil was carried out at several stages such as pre-formulations studies, evaluation and quantification of in-house developed polymeric nanoparticles and also for pharmacokinetic and biodistribution studies for the optimized formulations. Therefore, a simple, selective, rapid and cost-effective high-pressure liquid chromatography (HPLC) method was developed in-house to meet our need for both formulations

and biological samples and validated accordingly. All the developed methods were validated and are in compliance with the regulatory guidelines [3–5].

3.3 Chemicals and reagents

Donepezil and Loratadine standards were kind gift samples from Vasudha Pharma Chem Limited (Hyderabad, India). Acetonitrile, methanol (HPLC grade), mannitol, sucrose and sodium phosphate were procured from Merck Limited (Mumbai, India). Ammonium formate, polysorbate 80, trehalose, polyvinyl alcohol, polycaprolactone, polylactic acid was procured from Sigma Aldrich (St. Louis, MO, USA). Di-block co-polymer (mPEG-PCL) was synthesized in-house. Solid-phase cartridges, Clearert PEP-3 SPE was procured from Agela Technologies (Wilmington, USA). Triple distilled water was obtained from Millipore Milli-Q Plus water system (Millipore Bedford Corp., Bedford, MA, USA).

3.4 HPLC analytical method for estimation of donepezil

3.4.1 Instrumentation and chromatographic conditions

The liquid chromatography was carried out on a Shimadzu prominence HPLC system (Kyoto, Japan) consisting of a binary pump (LC-10AT), photo diode array (PDA) detector (SPD-M20A), column oven (CTO-10AS) and auto sampler (SIL-HTA, Shimadzu, Kyoto, Japan). Data acquisition and controlling the system was ascertained by LC solution software (Version 1.24 SP1). Chromatographic separation was attained at 30 ± 0.5 °C using a Waters Nova-Pak C18 column (3.9 x 150 mm, 4 μ m). The binary mobile phase consisted of acetonitrile and 0.01 M ammonium formate (pH 6.4) (30:70% v/v) at a flow rate of 1 mL/min in an isocratic mode. The aqueous phase was filtered through 0.22 μ m nylon membrane filter and ultrasonically degassed prior to use. The detections were carried out with injection volume of 20 μ L at a wavelength of 268 nm.

3.4.2 Method development

We tried with different types of columns, solvents and buffers, but the peak was showing better selectivity and sensitivity at Acetonitrile and 0.01 M ammonium formate (pH 6.4) (30:70) with 1.0 mL/min flow rate (isocratic condition) by using Waters Nova-Pak C18 column (3.9 x 150 mm, 4 μ m) in Shimadzu system SPD-M20A PDA detector.

3.4.3 Preparation of stock solution, calibration standards and quality control (QC) standards

The primary stock solution of donepezil (1 mg/mL) was prepared by solubilizing 5 mg of drug in 5 mL of 1:1 ratio of acetonitrile and water. From this primary stock solution, the working standards

in the range of 1-500 µg/mL were prepared by appropriately diluting the stock solution with mobile phase from higher to lower concentrations. Further, the calibration standards were prepared by appropriate dilution technique in the range of 0.1-50 µg/mL prepared in mobile phase. The QC samples were prepared by utilizing a respective working standard solution at three different levels i.e., low QC (LQC, 0.25 µg/mL), medium QC (MQC, 5 µg/mL) and high QC (HQC, 40 µg/mL).

3.4.4 Method validation

The developed analytical method was validated as per the ICH guidelines for specificity, selectivity, linearity, range, limit of detection, limit of quantification, accuracy, precision and robustness [4].

System suitability parameters were assessed before validating the method. For this, donepezil solution at a concentration of 5 µg/mL was injected six times and evaluated for its injection precision and other chromatographic parameters like tailing factor (Tf), capacity factor (k'), number of theoretical plates (N) and height equivalent to the theoretical plates (HETP).

Specificity and selectivity of the method was estimated by spiking expected formulation excipients like polysorbate 80, mannitol, sucrose, trehalose, polyvinyl alcohol, polycaprolactone, polylactic acid and di-block co-polymer (mPEG-PCL) in donepezil solution (5 µg/mL). These samples were analyzed through HPLC. A blank sample of placebo mixture was also analyzed to determine any interference with the retention time of donepezil.

Linearity and range of the calibration standards were prepared in triplicate in the range of 0.1-50 µg/mL. The best-fit calibration curves of the peak area versus the concentration were plotted and the data was analyzed by using least square regression analysis technique. Limit of detection (LOD) and limit of quantification (LOQ) was estimated by utilizing signal to noise (S/N) ratio method. Accuracy of the method was assessed at low, medium and high quality control samples of the analyte and were represented in terms of % bias and % recovery respectively. Further, the accuracy of the method was confirmed by spiking quality control standards in placebo sample and the accuracy of the sample was represented as % recovery.

Precision studies were carried out to determine repeatability and intermediate precision by estimating intra and inter day variations in sample analysis. Quality control standards were prepared in triplicates thrice a day for inter day analysis and the same protocol was followed for three consecutive days for intraday analysis. Precision data was represented in terms of %RSD of the predicted concentration from the co-efficient of regression equation.

Robustness of the method was performed to estimate the significance of minor variations in chromatographic conditions which may occur during day to day routine analysis. Robustness was estimated by making minor modifications in the chromatographic conditions like variation in the mobile phase composition, pH of the mobile phase and column oven temperature. Quality control standards were prepared and analyzed in triplicates with these minor changes and the results were represented as % bias and % RSD respectively.

Solution stability studies were conducted for quality control standards by storing at room temperature for 24 h and at refrigerator 4 °C for a period of one month (samples were analyzed in regular intervals). Further, the stability of the donepezil solutions was tested by forced degradation studies like acidic, alkaline, oxidation, thermal and photostability conditions. In brief, donepezil (50 µg/mL) samples were prepared in 0.1 N hydrochloric acid (HCl), 0.1 N sodium hydroxide (NaOH) and 5 % hydrogen peroxide, refluxed for 6 h at 60 °C. For thermal degradation analysis the drug sample was placed at temperature controlled hot air oven at 80 °C for 6 h and for photostability conditions donepezil samples were exposed to solar and UV radiations for 12 h each. Further, all the samples were allowed to cool at room temperature, diluted according and samples were analyzed. Results were represented as % bias and % recovery respectively.

3.4.5 Results and Discussion

Method development strategies were followed to obtain the optimum parameters that was both simple and economical. The major parameters considered during the method development was related to peak properties like retention time, tailing factor, peak shape, symmetry with insight to achieve higher sensitivity in terms of analyte peak area. For this, we have studied with different columns, buffers, pH of buffers and choice of organic solvents. Variations in pH (3-8) and buffer of mobile phase (acetate, formate, phosphate) showed minimal effect on peak properties of donepezil, may be due to unionized nature of molecule. Although, 0.01 M ammonium formate at pH 6.4 was selected further for validation procedure as it resulted in good peak properties, symmetry and higher sensitivity of donepezil. Methanol showed broader peaks and asymmetry, whereas utilization of acetonitrile resulted in satisfactory peak properties with higher sensitivity. After successive optimization, mobile phase composition of acetonitrile and 0.01 M ammonium formate (pH 6.4) at a ratio of 30:70 v/v in an isocratic mode with a flow rate of 1mL/min was selected which gave symmetrical peak shape, good peak properties and higher peak intensities. Initially, we have tried with different manufactures of reversed phase C18 columns such as Waters,

Agela technologies, Inertsil and Phenomenex for detection. Unfortunately, all of the columns showed longer retention time (more than 15 min) with peak broadening except Waters. This may be due to stronger affinity of donepezil with these columns. Therefore, we selected Waters Nova-Pak C18 column (3.9 x 150 mm, 4 μ m) column to get better peak shape, selectivity and retention time (fast analysis). The injection volume was 20 μ L and optimized retention time for donepezil was found to be 3.32 ± 0.1 min (Fig.3.1). The overall run time was 6 min and detection wavelength selected as 268 nm.

By performing the system suitability test, we conformed that system was working fine and it showed reproducibility and proper peak shape. With respect to system performance the developed method was found to be suitable and the results obtained for system suitability parameters like capacity factor ($k' \sim 1.2$), number of theoretical plates (~ 7464) and HETP (~ 21) were found to be within the acceptable limits. The developed method was found to be specific and selective as no interference from the placebo mixture was noticed in the vicinity of the drug peak. This conformed that the developed method was selective and suitable for routine analysis for the quantification of donepezil in nano-formulations in the presence of excipients (Fig.3.1). Moreover, in donepezil spiked placebo mix samples, no significant changes were observed in percent recovery and peak shape properties in comparison to the quality control standards.

The calibration standard curve showed reproducibility over the selected concentration range of 0.1 – 50 μ g/mL with a regression co-efficient (R^2) value above 0.999. The R^2 values, intercepts and slopes were estimated from five intra and inter day calibration curves. Calibration curve for donepezil in the optimized chromatographic conditions is shown in Fig.3.2. All the values were within the recommended limits.

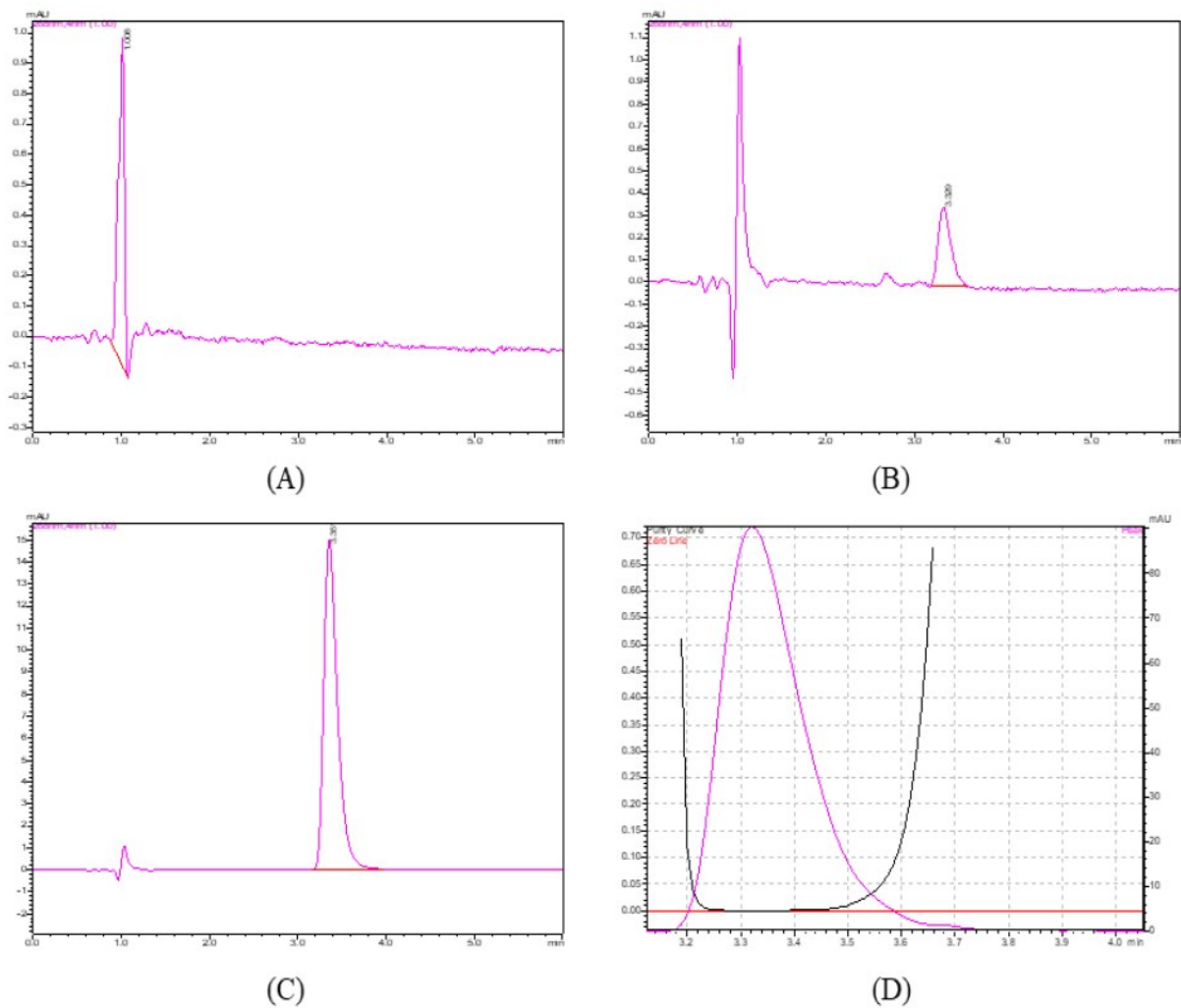


Fig. 3.1: Representative Chromatograms of donepezil indicating selectivity of the developed HPLC method (A) placebo sample (B) LQC sample (C) in-house developed nano-formulations sample (D) Peak purity index of analyte

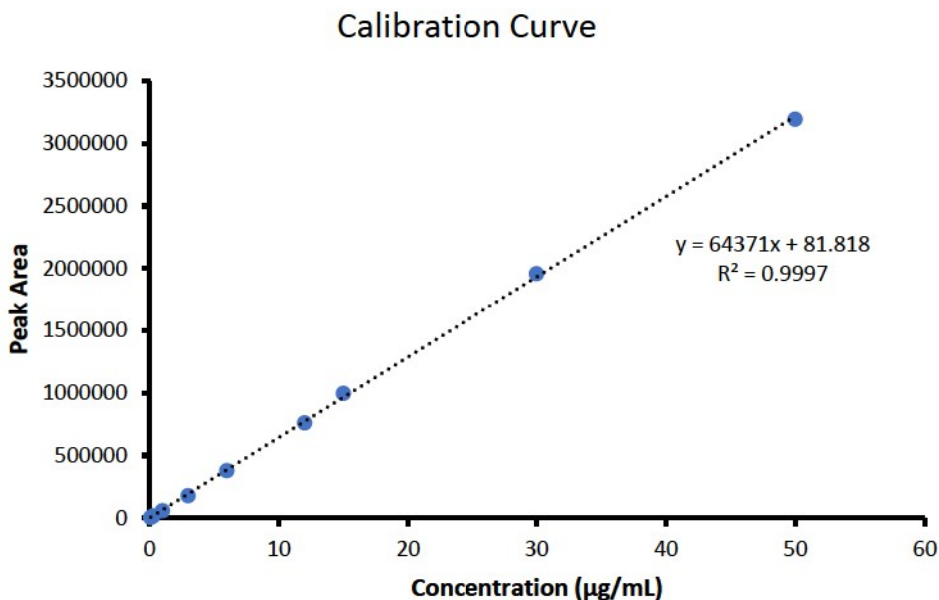


Fig. 3.2: Calibration curve of donepezil

Standard deviation (SD) and percent relative standard deviation (%RSD) values are within the acceptable limits at all concentration levels and the calibration data is shown in the Table 3.1.

Table 3.1 Calibration curve data for the quantification of donepezil by HPLC

Concentration ($\mu\text{g/mL}$)	Peak area \pm SD	% RSD
0.10	6113.00 \pm 41.33	1.37
0.30	18354.33 \pm 36.23	0.40
1.00	60340.33 \pm 97.35	0.65
3.00	178618.33 \pm 97.35	0.25
6.00	379190.00 \pm 458.03	1.21
12.00	762838.67 \pm 410.99	0.22
15.00	996917.00 \pm 734.31	1.47
30.00	1958500.67 \pm 691.32	1.29
50.00	3197027.67 \pm 864.62	1.48

SD, standard deviation; % RSD, percent relative standard deviation

The LOD and LOQ were found to be 40 and 100 ng/mL respectively which are determined by using S/N method.

The accuracy and precision of the developed method was estimated for all the quality control standard samples. The deviation in intra and inter-day precision (%RSD) at three QC samples

(LQC, MQC and HQC) were in the range of 0.18 to 1.82 %. Whereas, accuracy (% bias) was found to be in the range of -6.50 to 4.06. Based on the values, it was conformed that the variations were within the acceptable limits as per recommended guidelines. The data for inter and intra-day precision and accuracy is being represented in Table 3.2. Moreover, the accuracy of the developed method was confirmed by spiking donepezil QC standards in placebo mix. Data obtained resulted in high recovery of about 100 % representing the stability of the method for quantification of donepezil in the presence of nano-formulation excipients and results were tabulated in Table 3.3.

Table 3.2 Accuracy and Precision data for the developed analytical method

QC Standard	Nominal Concentration (µg/mL)	Predicted Concentration (µg/mL)		% Recovery ± SD	% Bias
		Mean ± SD	% RSD		
Intra-day (n=9)					
LQC	0.25	0.23 ± 0.01	0.60	93.74 ± 0.56	-6.26
MQC	5.00	5.20 ± 0.09	1.82	104.06 ± 1.89	4.06
HQC	40.00	37.40 ± 0.07	0.19	93.50 ± 0.18	-6.50
Inter-day (n=27)					
LQC	0.25	0.24 ± 0.01	0.99	94.52 ± 0.93	-5.48
MQC	5.00	5.11 ± 0.05	0.89	102.28 ± 0.91	2.28
HQC	40.00	39.00 ± 0.11	0.28	97.50 ± 0.27	-2.50

QC Standard, quality control standard; SD, standard deviation; % RSD, percent relative standard deviation; LQC, MQC and HQC are low, medium and high level quality control (QC) standards of donepezil respectively

Table 3.3 Accuracy studies for HPLC analytical method by placebo spiking method

Amount of donepezil spiked (µg/mL)	Recovered Concentration (µg/mL) ± SD	% Recovery ± SD	% Bias
0.25	0.24 ± 0.01	96.14 ± 1.20	-3.86
5.00	5.13 ± 0.06	102.65 ± 1.21	2.65
40.00	39.14 ± 0.29	97.8 ± 0.72	-2.14

SD, standard deviation; % RSD, percent relative standard deviation

Robustness of the developed analytical method was carried out with minor alterations in chromatographic condition. The method was found to be robust and no significant changes were noticed in peak area and peak shape even the chromatographic conditions deviated from the

optimized one with respect to pH of mobile phase (6-7), acetonitrile composition in mobile phase (35-25%) and column oven temperature (25-35 °C). The percent recovery of donepezil was found to be in the range of 96.03 ± 0.97 to $103.42 \pm 0.81\%$ and the results were represented in Table 3.4. Solution stability for QC standards was performed at ambient temperature (25 °C) for about 24 h and at 4 °C for a month. Stability of the samples were estimated based on the peak areas with respect to freshly prepared QC samples. The data was represented in terms of percent recovery which was in the range of 96.98 – 101.25%. These results confirmed that the developed method was specific, accurate, precise and robust for the quantification of donepezil and its nano-formulations.

Table 3.4 Robustness data of the developed HPLC analytical method

Parameters Changed		% Recovery \pm SD			% RSD		
		LQC	MQC	HQC	LQC	MQC	HQC
Acetonitrile composition in mobile phase (v/v)	35:65	96.14 \pm 1.20	102.65 \pm 1.20	97.86 \pm 0.72	1.24	1.17	0.74
	25:75	96.58 \pm 1.72	101.87 \pm 1.73	98.68 \pm 1.89	1.78	1.70	1.92
pH of buffer	6	96.04 \pm 0.71	102.30 \pm 0.56	98.80 \pm 1.80	0.74	0.54	1.82
	7	97.23 \pm 0.52	103.42 \pm 0.81	101.21 \pm 1.57	0.54	0.78	1.55
Column temperature (°C)	25	96.03 \pm 0.97	101.59 \pm 1.75	100.03 \pm 1.49	1.01	1.73	1.49
	35	97.14 \pm 1.63	101.94 \pm 1.80	99.65 \pm 1.49	1.67	1.77	1.49

Forced degradation studies were carried out for donepezil and the recovery was found to be in the range of 94.15 – 98.18 % except in oxidation and hydrolysis in basic conditions. In both the cases, decrease in peak intensities of donepezil was noticed which indicated the degradation nature of DNP in alkaline conditions and the results were represented in Table 3.5 which are in accordance with the earlier reports. However, no interference or any additional peaks were detected. The

representative chromatograms are shown in Fig.3.3. This indicates the developed method was suitable for routine analysis and selectively was not affected.

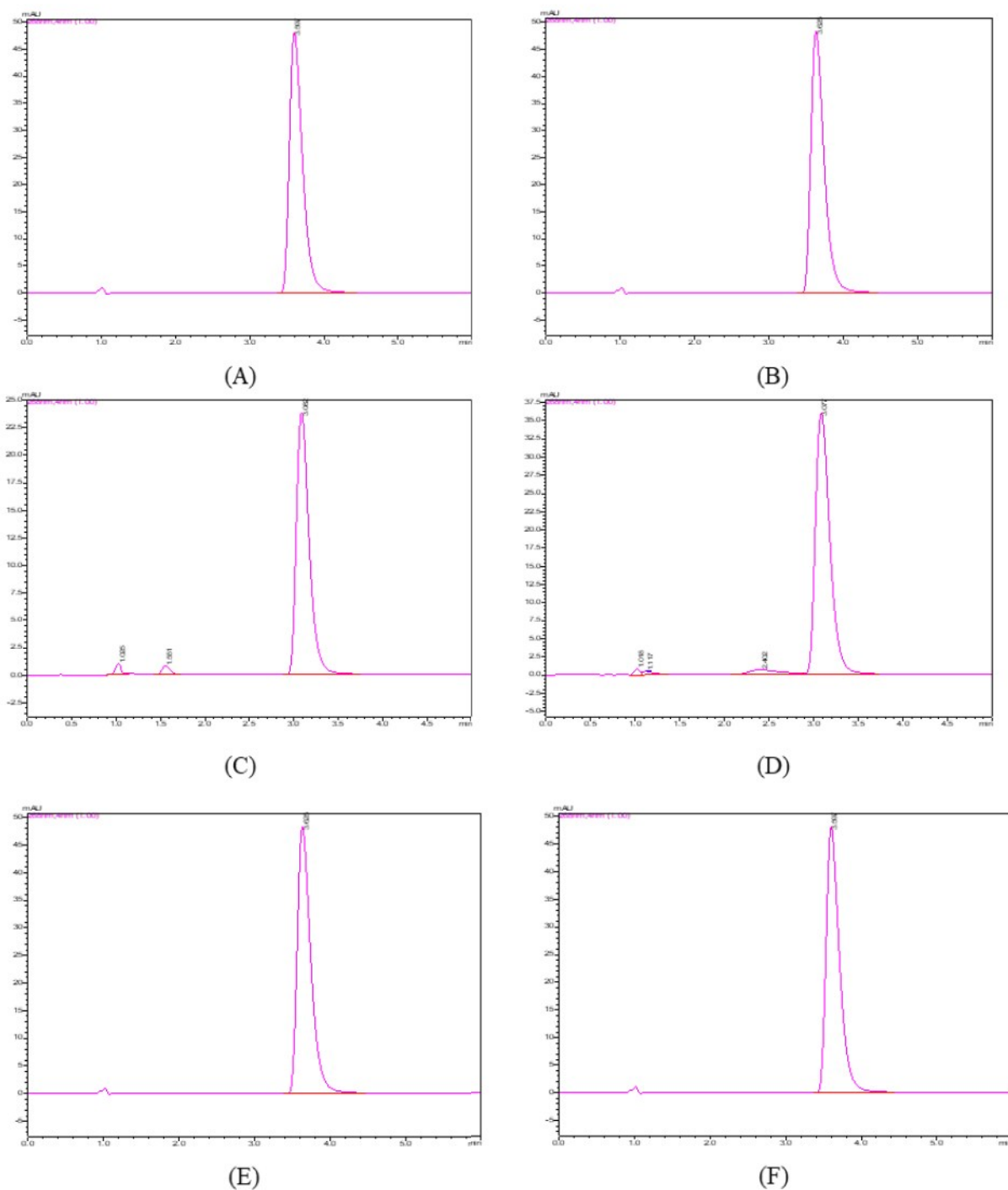


Fig.3.3: Representative chromatograms of stress induced donepezil samples; (A) standard donepezil sample; (B) acid hydrolysis; (C) base hydrolysis; (D) oxidation; (E) photostability; (F) thermal stress

Table 3.5 Forced degradation studies of donepezil

Stress Condition	% Recovery \pm SD (n=3)
Acid hydrolysis (0.1 N HCl at 60 °C, 4 h)	98.17 \pm 2.15
Base hydrolysis (0.1 N NaOH at 60 °C, 4 h)	58.65 \pm 3.14
Oxidation (5% hydrogen peroxide at 60 °C, 4 h)	52.44 \pm 2.98
Photostability (UV + Solar, 12 h each)	99.24 \pm 1.92
Thermal (80 °C, 4h)	98.31 \pm 2.63

Table 3.6 Summary of validation parameters of donepezil analytical method

Parameter	Value
Calibration range	0.1 – 50 μ g/mL
Regression coefficient	$R^2 = 0.9997$
Regression equation	$y = 64371x + 81.818$
Limit of detection (LOD)	0.04 μ g/mL
Limit of quantification (LOQ)	0.1 μ g/mL
Absolute recovery	96.14 – 102.65 %
Accuracy (% bias)	-6.50 – 4.06 %
Precision (% RSD)	0.19 – 1.82 % (Intraday) 0.28 – 0.99 % (Interday)
System suitability	$k' \sim 1.2$, $N \sim 7464$, HETP ~ 21
Selectivity	Selective
Robustness	No significant changes were observed in the chromatographic parameters on making minor changes

The analytical method for donepezil was successfully developed and validated which was used to determine the percent entrapment efficiency and drug loading for in-house developed nano-formulations. These results were discussed in Chapter 5.

3.5 Bio-analytical method for quantification of donepezil in rat plasma and tissues by HPLC

3.5.1 Instrumentation and chromatographic conditions

HPLC analysis was performed on Shimadzu liquid chromatograph system (Kyoto, Japan) consisted of a binary pump (LC10AT), PDA detector (SPD-M20A), column oven (CTO-10AS)

and auto sampler (SIL-HT, Shimadzu, Japan) was utilized to inject the samples. Analytes were observed at a wavelength of 268 nm using a PDA detector. Donepezil and loratadine (internal standard) were separated on Waters Nova-Pak C18 column (3.9 x 150 mm, 4 mm) with a mobile phase containing acetonitrile and ammonium formate (pH 6.4; 0.01 M) (62:38% v/v) at a flow rate of 1 mL/min in an isocratic mode and at a column oven temperature of 40 ± 0.5 °C. The injection volume was 50 μ L and retention time for donepezil and loratadine were 3.31 and 3.99 min respectively. Data integration and hardware control was carried out by utilizing LC solution software version 1.24 SP1.

3.5.2 Collection of plasma and tissue homogenates

All experimental animals (Wistar rats; male; 8–10 weeks; weighing around 200–220 g), were housed in polyacrylic cages, and the experimental procedure was performed as per the guidelines of the Institutional Animal Ethics Committee (IAEC) of BITS-Pilani university with approved protocol (IAEC/RES/22/05). All animals were kept at central animal facility and subjected to 12/12 h light/dark cycle with a controlled temperature (20 – 22 °C) and humidity (65%). Both feed and water were provided *ad libitum* throughout the acclimatization and study period. Blood samples were obtained from the retro-orbital plexus at selected time intervals. Plasma samples were collected by centrifuging the blood at 6000 rpm for 15 min and stored at -80 ± 10 °C until analysis.

Tissues (brain, heart, lungs, kidney, spleen and liver) were promptly harvested at selected time intervals (three rats/time point) after the administration of dose and rinsed thoroughly with ice-cold saline to remove any blood or other components and blotted with filter paper. A precisely weighed quantity of each tissue samples were homogenized at 20000 rpm by using Kinematica™ Polytron™ PT 1600E benchtop homogenizer in 1:2 w/v of phosphate buffer saline solution by using tissue homogenizer. After homogenization, centrifugation was done at 10000 rpm for 15 min, clear supernatant was collected and stored at -80 ± 10 °C until analysis.

3.5.3 Preparation of stock solutions, calibration curve standards and quality control sample

Standard stock solution of donepezil was prepared in methanol to attain a final concentration of 5 mg/mL. Further, same volumes of donepezil stock solution (5 mg/mL) was diluted with Milli-Q water to make 400 μ g/mL working stock solution. From this, working standard solutions of 1, 2, 6, 20, 40, 70 and 100 μ g/mL and working QC standard solutions of 1.6, 30 and 80 μ g/mL were prepared by serial dilutions in Milli-Q water. The calibration standards and QC samples were

prepared by spiking with 10 μL of respective working standards in 190 mL of blank rat plasma or tissue sample. The final calibration standards were prepared as 50, 100, 300, 1000, 2000, 3500 and 5000 ng/mL in plasma matrix. The QC samples were prepared by utilizing QC standards at four concentration levels, such as limit of quantification (LOQQC, 50 ng/mL); low QC (LQC, 80 ng/mL), medium QC (MQC, 1500 ng/mL) and high QC (HQC, 4000 ng/mL). The working and stock standards were kept in refrigerated conditions at $-20\text{ }^{\circ}\text{C}$.

3.5.4 Sample preparation

Several extraction techniques were verified, like protein precipitation with organic solvents (methanol, acetonitrile) and by liquid–liquid extraction using dichloromethane, n-hexane: isopropyl alcohol (97:3) and n-hexane: isopropyl alcohol (95:5). These procedures allow good recovery for IS, but plasma interference was observed with low recovery of analyte.

Solid phase extraction (SPE) method was also tried to decrease the solvent utilization, achieve maximum and reproducible analytes recovery and optimize the sample clean-up process. A 200 μL aliquot of plasma or tissue sample comprising donepezil was taken in 2 mL capacity of polypropylene disposable tube, afterwards addition of 20 μL of 100.0 $\mu\text{g}/\text{mL}$ of loratadine (internal standard) solution (prepared in acetonitrile) and was vortexed for 1 min. Then 200 μL of ammonium formate buffer was added and vortexed for 2 min on a vortex mixer. The samples were then loaded into the pre-conditioned, equilibrated SPE cartridges using a semi-automated SPE system. The SPE eluents were collected in glass tubes and evaporated at $40\pm 0.5\text{ }^{\circ}\text{C}$ with a gentle stream of nitrogen gas and reconstituted with 200 μL of mobile phase. Finally, 50 μL was subjected to HPLC analysis for the quantification.

3.5.5 Method validation parameters

The developed method was validated as per guidelines US-FDA and European Medical Agency guidelines of bioanalytical method validation.

System suitability is typically a system performance test, which was carried out by injecting the same concentration sample by six times for checking the column efficiency, reproductivity and resolution. It was performed to verify the holistic function of the chromatography system on a day-to-day basis. The selectivity of the method was performed to estimate possible chromatographic interference from the rat plasma or tissue matrix at retention time of the drug and internal standard. The rat blank plasma and tissue samples were collected randomly from Wistar rats and were analyzed as discussed.

The calibration curve of an analyte was prepared by utilizing seven calibration standards in the range of 50 to 5000 ng/mL. Six calibration curves were generated for plasma and tissue samples by plotting plasma drug concentrations on X-axis and peak area ratios of drug by IS on Y-axis. Highest calibration standard sample (ULOQ, 5000ng/mL) was 100 times less than the lowest limit of calibration point (LLOQ, 50ng/mL), so it was necessary to perform weighted linear regression on calibration data. All the various weighing factors were determined to estimate the best weighing factors based on percent relative error (%RE) from the least-squares regression analysis.

The limit of detection (LOD) is defined as the lowest concentration of analytes that can be detected and differentiated from the noise level. The lower limit of quantitation (LLOQ) is defined as the lowest concentration that could be quantified with accepted accuracy and precision recommendations (not exceeding ±20%). LOD and LLOQ were estimated by using signal to noise ratio method. First the signal to noise ratio was checked for standard samples at system suitability step. LOD and LLOQ were calculated based on equation 1. Desired S/N values for LOD was 3 and for LLOQ was 10.

$$\text{Concentration of LOQ and LOD} = \frac{\text{Concentration of Standard}}{\frac{S}{N} \text{ value of Standard}} \times \text{Desired } \frac{S}{N} \text{ value} \dots \text{eq.1}$$

Generally, LLOQ is the first point in calibration curve and analyzed plasma or tissue samples by spiking with LLOQ concentration in six replicates (n=6).

The intra-day and inter-day accuracy and precision were estimated by analyzing at three different QC levels (LQC, MQC, HQC) as described in section 3.5.3 and LLOQ. The intra-day accuracy and precision were estimated in six replicates by analyzing samples on same day, while inter-day accuracy and precision were estimated in six replicates by analyzing each QC and LLOQ samples on five different days. The acceptable criteria for intra and inter-day precision (RSD, %) and accuracy were limited to ±15% except for LLOQ, where it should not exceed ±20% for precision and accuracy.

The percentage recovery of donepezil through SPE were estimated by comparing the peak area of analyte spiked plasma QC samples with those of unextracted neat standards that is standard solution with equivalent concentrations.

Carry over effect was estimated by injecting highest calibration standard sample (ULOQ, 5000 ng/mL) next to blank sample. As per guidelines, the sample dilution must be performed to ensure the integrity of plasma samples. In dilution integrity, blank plasma was spiked with 2 times higher than the ULOQ concentration (10000 ng/mL) and was diluted 5 times and 10 times with blank

plasma (n=6) to obtain a concentration of 2000 ng/mL and 1000 ng/mL. Dilutes samples were processed and analyzed with freshly spiked calibration curve in plasma. The accuracy and precision values should be within $\pm 15\%$.

Stability of analytes in plasma and tissue matrices were evaluated before and after exposing to various conditions and temperatures that might encounter in regular sample analysis. Stability of the analyte was estimated in terms of bench top stability, freeze–thaw stability, autosampler stability, short-term stability and long-term stability. All these studies were performed in triplicates at three different QC levels (LQC, MQC, and HQC). Bench top stability was carried out at 25 ± 2 °C for 24 h, freeze thaw stability was conducted after freezing (-80 ± 10 °C for 48 h) and thawing QC samples for three consecutive cycles. Autosampler stability was conducted for 48 h, short-term stability was evaluated at -80 ± 10 °C for 15 days and long-term stability was examined at -80 ± 10 °C for 3 months were performed in rat plasma or tissue samples with all QC levels. All QC samples were estimated against freshly prepared calibration curve samples and the acceptance criteria for accuracy and precision for all these stability conditions must be within $\pm 15\%$

3.5.6 Analysis of *in vivo* samples

Pharmacokinetic and biodistribution samples of both pure drug and in-house developed nano-formulations were analyzed by using above validated method. The procedure and results were discussed in Chapter 6.

3.5.7 Results and Discussion

A methodological approach was utilized for the method development to attain analyte in less time using simplest possible chromatographic conditions with an appreciable recovery, symmetric peaks and good resolution of the drugs. For that, liquid chromatographic conditions like selection of mobile phase, composition of mobile phase, pH of buffer, flow rate, column selection and injection volume were optimized (Table 3.7). After successive optimization, acetonitrile-ammonium formate (pH:6.4; 0.01 M) was selected as mobile phase which gave best peak shape and good peak intensities. Different columns were used to optimize better resolution, selectivity and retention time. Waters Nova-Pak C18 column (3.9×150 mm, $4\mu\text{m}$) with acetonitrile-ammonium formate (pH:6.4; 0.01M) mobile phase in ratio of 62:38 % v/v at 1 mL/min flow rate in an isocratic mode was found to be most suitable for donepezil analysis with a total run time of 6 min. The injection volume was 50 μL and optimized retention time for donepezil and loratadine were 3.31 and 3.99 min respectively at a wavelength 268 nm.

Once the chromatographic conditions were optimized, extraction recovery of analyte was optimized by selecting an appropriate extraction method. The plasma sample clean-up process was also an important step in the development of method that is directly proportional to selectivity and sensitivity of method. Maximum recovery of analyte from plasma matrix might be attained by minimum sample processing and proper choice of extraction method. Various problems have been encountered during the method optimization. Different methods for extracting drug from plasma matrix have been tried such as protein precipitation (with acetonitrile and methanol), liquid-liquid extraction (with hexane, methylene chloride, isopropyl alcohol) and SPE (Table 3.8). Out of all, SPE was found to be the most appropriate sample preparation technique with higher recoveries, reproducibility and cleaner chromatogram.

Table 3.7 Method optimization: System suitability parameters obtained upon performing deliberate variations in the chromatographic conditions

Variations in Chromatographic condition	Effect on chromatographic parameters of analytes (n=6)												[‡] Resolution n
	t _r (min)			Tailing factor (10%)			HETP			N			
	DNP	IS	IS	DNP	IS	IS	DNP	IS	IS	DNP	IS	IS	
No variation ([‡] Optimized)	3.31 ± 0.01	3.99 ± 0.01	1.05 ± 0.06	1.14 ± 0.04	69.96 ± 6.44	30.20 ± 1.09	2431.83 ± 143.65	4993.86 ± 187.30	2.88 ± 0.09				
Flow rate (0.8 mL/min)	4.25 ± 0.02	5.01 ± 0.01	1.22 ± 0.08	1.27 ± 0.01	75.22 ± 17.72	29.77 ± 0.27	2076.87 ± 175.78	5037.57 ± 46.35	2.30 ± 0.16				
Flow rate (1.2 mL/min)	2.80 ± 0.02	3.32 ± 0.01	1.28 ± 0.10	1.19 ± 0.02	86.25 ± 12.71	38.15 ± 0.45	1528.87 ± 219.47	3931.61 ± 47.57	1.49 ± 0.32				
Mobile phase composition (60:40 % v/v)	3.49 ± 0.01	4.43 ± 0.02	1.22 ± 0.17	1.18 ± 0.01	68.71 ± 6.57	31.49 ± 0.28	2198.00 ± 209.92	4763.50 ± 43.90	3.35 ± 0.12				
Mobile phase composition (64:36 % v/v)	3.18 ± 0.02	3.71 ± 0.18	1.15 ± 0.07	1.18 ± 0.02	82.25 ± 20.70	36.42 ± 0.32	1275.77 ± 172.56	4118.09 ± 34.34	1.46 ± 0.34				
Column temperature (35 °C)	3.55 ± 0.04	4.08 ± 0.04	1.31 ± 0.09	1.28 ± 0.04	56.01 ± 11.94	34.40 ± 0.32	2772.14 ± 88.70	4360.73 ± 41.14	2.02 ± 0.02				
Column temperature (45 °C)	3.33 ± 0.08	3.90 ± 0.02	1.36 ± 0.07	1.29 ± 0.02	45.71 ± 1.04	32.97 ± 0.38	2793.95 ± 61.73	4549.15 ± 53.31	2.36 ± 0.06				
Buffer (pH:4.4)	2.69 ± 0.12	3.99 ± 0.09	1.37 ± 0.09	1.24 ± 0.04	78.75 ± 2.39	33.44 ± 0.09	1921.48 ± 135.36	4485.23 ± 52.85	5.35 ± 0.08				
Buffer (pH:8.4)	3.53 ± 0.16	3.97 ± 0.11	1.34 ± 0.14	1.27 ± 0.09	48.75 ± 2.87	30.50 ± 0.95	3079.95 ± 244.04	4965.65 ± 31.81	1.89 ± 0.18				

[‡]Optimized; Flow rate-1 mL/min, Mobile phase composition (62:38 % v/v), Column temperature (40 °C), Buffer pH (6.4)

[‡]Resolution; Resolution factor for DNP and IS peak

t_r, retention time; HETP, height equivalent to theoretical plate; N, number of theoretical plates

Table 3.8 Optimization of Solid-Phase Extraction (SPE) method for sample preparation

Extracting technique	Extracting solvent	Sample volume (μL)	Volume of extraction solvent added (mL)	Vortex time (min)	Centrifugation [speed (rpm), time (min)]	% Recovery	Remarks
Protein Precipitation	Acetonitrile	200	3	5	5000, 15	35-42%	Poor recovery, interference of plasma was observed and inconsistent recovery
	Methanol	200	3	5	5000, 15	40-49%	Poor recovery, interference of plasma was observed and inconsistent recovery
Liquid-Liquid extraction	Methylene chloride (DCM)	200	3	5	5000, 15	25-37 %	Very poor recovery and high interference of plasma was observed
	n-hexane	200	3	5	5000, 15	64-72 %	Increase in the recovery of analyte was observed, but recovery was inconsistent and high plasma interference was observed
	n-hexane: IPA (97:3 v/v)	200	3	5	5000, 15	65-79 %	Slight increase in the recovery of analyte was observed, but recovery was inconsistent and high plasma interference was observed
	n-hexane: IPA (95:5 v/v)	200	3	5	5000, 15	68-78%	Similar recovery of analyte was observed, but recovery was inconsistent and high plasma interference was observed
Solid-Phase extraction (SPE)	Acetonitrile	200	2	2	--	49-64%	No interference of plasma proteins was observed but recovery was reduced
	Methanol	200	2	2	--	70-78%	Consistent recovery was observed, no interference of plasma proteins was observed
	Methanol: 10 mM Ammonium formate (pH:6.4) (90:10 v/v)	200	2	2	--	66-72%	Slight decrease in the recovery of analyte was observed, no interference of plasma proteins was observed
SPE		100	2	2	--	75-76 %	LLOQ was high (in $\mu\text{g/mL}$)
	Methanol	200	2	2	--	77-79 %	LLOQ was improved (in ng/mL)
		300	2	2	--	78-80%	Consistent recovery and reproducible with no plasma interference, LOD was observed at 20 ng/mL , I.S. recovery was also good and consistent

Based on the above results, SPE was selected as the extracting technique and methanol as solvent of choice for elution and other extraction conditions (sample volume) were further optimized

By performing the system suitability test, we confirmed that system was working fine and it showed reproducibility with proper resolution. In described chromatographic condition, analytes were successfully separated with optimum retention time less than 6 min. The sample clean-up procedure was so selective as there was no significant plasma matrix interference at retention time of analyte. A representative chromatogram of blank plasma, (plasma sample without analyte and IS), plasma sample with analyte and IS, LOD plasma sample (20ng/mL) and LLOQ plasma sample (50 ng/mL) are shown in Fig.3.4. The representative chromatograms for tissue samples are shown in Fig.3.5.

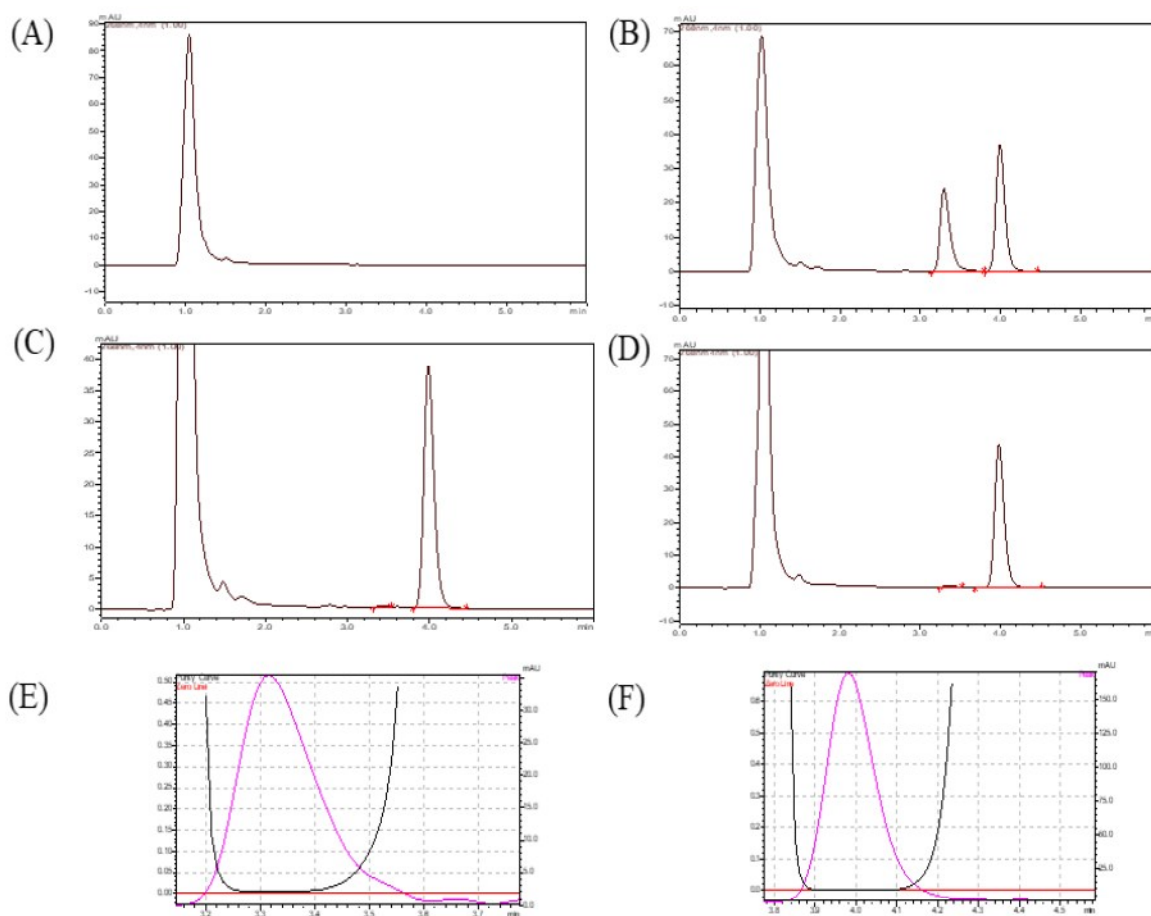


Fig. 3.4 HPLC Chromatograms for (A) Blank plasma (B) Calibration standard (C) LOD (20ng/mL) (D)LLOQ (50ng/mL) (E) Peak purity index of DNP (F) Peak purity index of IS

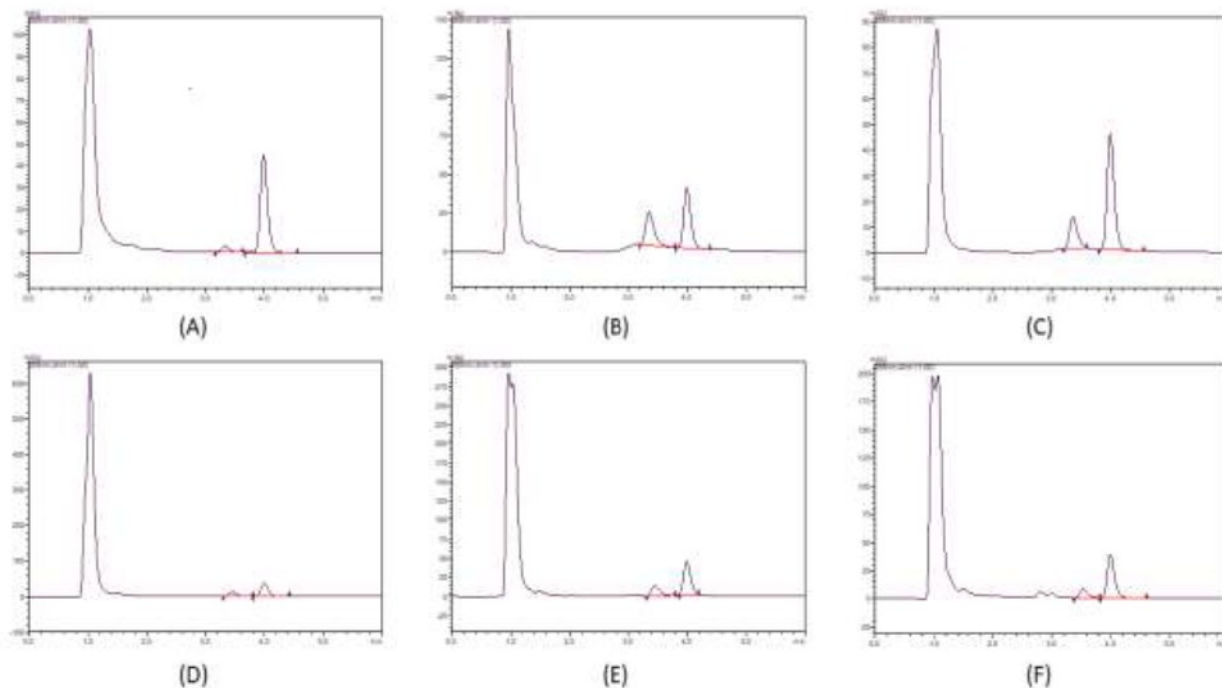


Fig. 3.5: Representative HPLC Chromatograms for (A) Brain (B) Spleen (C) Heart (D) Kidney (E) Lungs (F) Liver. All the calibration standard curves for plasma and tissue samples showed reproducibility and linearity over the selected concentration range of 50-5000 ng/mL with R^2 values more than 0.9999. The R^2 values, intercepts and slopes were estimated from five intra and inter day calibration curves by utilizing weighted linear regression analysis ($1/\text{var}$, $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$, $1/\sqrt{y}$) wherein, best weighting factors were chosen as $1/x^2$ as these showed least percentage relative error (% RE) (Table 3.9). The observed mean back calculated concentrations with accuracy (%bias) and precision (%CV) are represented in Table 3.10. At LLOQ ($n = 6$) accuracy (%bias) was in the range of 3.74 % with a %CV of 6.19 %. LOD and LLOQ were established as 20 ng/mL and 50 ng/mL respectively.

Table 3.9 Regression parameters of the calibration curve generated for each weighting factor (w_i) and their respective sum of the relative errors ($\Sigma \%RE$)

Model (w_i)	b	a	r^2	$\Sigma \%RE$
Unweighted	0.000400	-0.0114	0.9999	-5.6094
$1/\text{var}$	0.000383	-0.0082	0.9997	3.9284
$1/x^2$	0.000384	-0.0088	0.9999	0.5904
$1/x$	0.000382	-0.0083	0.9995	0.7194
$1/x^{1/2}$	0.000384	-0.0093	0.9999	4.1923
$1/y^2$	0.000380	-0.0081	0.9993	0.7303

1/y	0.000384	-0.0087	0.9999	1.4182
1/y ^{1/2}	0.000384	-0.0092	0.9999	3.2725

b, slope; *a*, constant; *r*², regression co-efficient

Table 3.10 Precision and accuracy data of back calculated concentrations of calibration standard samples of donepezil in rat plasma and various tissues (n = 6)

Component	Linear equation and Coefficient of regression	Nominal concentration (ng/mL)	Measured concentration (Mean ± SD, ng/mL)	Precision (% CV)	Accuracy (% bias)
Plasma	y=0.000384x-0.0088 r ² = 0.9999	50	51.87 ± 3.21	6.19	3.74
		100	95.30 ± 4.57	4.80	-4.70
		300	304.26 ± 16.99	5.58	1.42
		1000	999.58 ± 2.96	0.30	-0.04
		2000	1983.08 ± 12.60	0.64	-0.85
		3500	3464.12 ± 5.27	0.15	-1.03
		5000	5042.00 ± 8.58	0.17	0.84
Brain	y=0.0003x-0.0058 r ² = 0.9998	50	55.26 ± 1.15	2.08	10.52
		300	289.54 ± 15.48	5.35	-3.49
		1000	939.40 ± 87.62	9.33	-6.06
		2000	1950.68 ± 13.60	0.70	-2.47
		3500	3335.07 ± 30.75	9.18	-4.71
		5000	4872.22 ± 19.75	0.41	-2.56
Heart	y=0.0003x-0.0075 r ² = 0.9991	50	56.24 ± 6.54	11.63	12.48
		300	314.36 ± 1.10	0.35	4.79
		1000	1011.78 ± 18.88	1.87	1.18
		2000	2147.01 ± 51.93	2.42	7.35
		3500	3547.65 ± 4.37	0.12	1.36
		5000	5262.01 ± 43.58	0.83	5.24
Lungs	y=0.0003x-0.0099 r ² = 0.9999	50	56.78 ± 4.23	7.45	13.56
		300	316.62 ± 28.83	9.11	5.54
		1000	1041.39 ± 93.03	8.93	4.14
		2000	2168.05 ± 106.81	4.93	8.40
		3500	3749.94 ± 206.25	5.50	7.14
		5000	5372.00 ± 291.26	5.42	7.44
Spleen	y=0.0003x+0.0089 r ² = 0.9996	50	55.31 ± 3.32	6.00	10.62
		300	289.15 ± 8.66	2.99	-3.62
		1000	1049.53 ± 80.29	7.65	4.95
		2000	2148.97 ± 146.01	6.79	7.44
		3500	3600.13 ± 269.69	7.49	2.86
		5000	5247.77 ± 32.88	0.63	4.96
Kidney	y=0.0003x-0.0008	50	46.42 ± 4.12	8.88	-7.16

	$r^2 = 0.9995$	300	309.21 ± 3.51	1.14	3.07
		1000	1050.37 ± 85.23	8.11	5.04
		2000	2243.01 ± 223.27	9.95	12.15
		3500	3804.79 ± 163.19	4.29	8.71
		5000	5355.65 ± 51.11	0.95	7.11
Liver	$y=0.0003x-0.0126$	50	52.94 ± 1.68	3.17	5.88
	$r^2 = 0.9999$	300	281.24 ± 25.88	9.20	-6.25
		1000	935.92 ± 29.60	3.16	-6.41
		2000	1880.22 ± 135.81	7.22	-5.99
		3500	3240.65 ± 18.66	0.58	-7.41
		5000	4703.54 ± 5.48	0.12	-5.93

SD, standard deviation; CV, coefficient of variation

The intra and inter day accuracy, precision data obtained from QC samples at different concentration levels (LLOQC, LQC, MQC, HQC) are shown in Table 3.11. The inter and intra-day precision values did not exceed 6.92 %. The inter and intra-day % bias ranged from -3.11 to 7.08 %. All the values were within the recommendation of international guidelines.

Table 3.11 Precision (% CV) and accuracy (% bias) of DNP in rat plasma samples at QC levels (n=6)

Level	Nominal Conc. (ng/mL)	Inter-day			Intra-day		
		Measured Conc. (Mean \pm SD, ng/mL)	Precision (% CV)	Accuracy (% bias)	Measured Conc. (Mean \pm SD, ng/mL)	Precision (% CV)	Accuracy (% bias)
LLOQ	50	53.54 ± 2.43	4.54	7.08	50.21 ± 3.19	6.35	0.42
LQC	80	79.74 ± 4.45	5.58	-0.33	77.51 ± 5.36	6.92	-3.11
MQC	1500	1543.40 ± 14.82	0.96	2.89	1513.29 ± 22.85	1.51	0.89
HQC	4000	4025.35 ± 25.97	0.65	0.63	3926.75 ± 65.42	1.67	-1.83

The mean recovery values from rat plasma samples are listed in Table 3.12. The absolute recovery of DNP was found to be 79.86 ± 1.55 % (n=12). Analyte peak was not detected in the blank sample injected after ULOQ, that conforms the absence of any carry over. The dilution integrity was carried out by diluting 5 times and 10 times with the blank plasma (n=6). The accuracy value after 5 times and 10 times dilution were found to be 98.60 ± 3.47 and 101.75 ± 2.81 respectively.

The stability studies for donepezil were carried out as per the procedure described in section 3.5.5. Different stability studies such as stock solution stability, bench top stability, autosampler stability, freeze–thaw stability and short-term stability results indicated that DNP was stable under various storage conditions which might be faced in routine sample analysis and there was no significant degradation observed (Table 3.13).

Table 3.12 Absolute recoveries (%) of donepezil in rat plasma samples from QC levels

Level	Nominal concentration (ng/mL)	n	Recovery (%)	
			Mean \pm SD (n=3)	% CV
LLOQ	50	3	80.22 \pm 1.44	1.80
LQC	80	3	80.28 \pm 1.18	1.47
MQC	1500	3	78.68 \pm 1.62	2.06
HQC	4000	3	80.28 \pm 1.95	2.43
	Mean	12	79.86 \pm 1.55	1.94

n, number of samples; SD, standard deviation; CV, coefficient of variation

Table 3.13 Stability studies for donepezil in rat plasma at QC levels

Stability	Nominal concentration (ng/mL)	Measured Concentration (ng/mL) \pm SD	Precision (% CV)	Accuracy (% bias)
0 h (for all)	50	53.54 \pm 2.43	4.54	7.08
	80	79.97 \pm 4.45	5.58	-0.33
	1500	1543.40 \pm 14.82	0.96	2.89
	4000	4025.35 \pm 25.97	0.65	0.63
Autosampler (48 h)	50	51.72 \pm 2.60	5.03	3.44
	80	83.48 \pm 4.06	4.86	4.35
	1500	1446.35 \pm 8.49	0.59	-3.58
	4000	3972.38 \pm 5.35	0.13	-0.69
Bench-top (24 h, RT)	50	54.12 \pm 1.42	2.62	8.24
	80	84.49 \pm 4.94	5.85	5.61
	1500	1548.91 \pm 7.95	0.51	3.26
	4000	3951.87 \pm 24.88	0.63	-1.20
Freez-thaw (-80 °C, 3 cycle)	50	54.84 \pm 2.50	4.56	9.68
	80	82.64 \pm 6.28	7.60	3.30
	1500	1463.00 \pm 15.13	1.03	-2.47
	4000	3840.44 \pm 18.39	0.48	-3.99
Short-term (-80 °C, 15 days)	50	49.74 \pm 2.68	5.39	-0.52
	80	78.01 \pm 2.62	3.36	-2.49

	1500	1414.08 ± 54.99	3.89	-5.73
	4000	3722.16 ± 12.29	0.33	-6.95
Long-term (-80 °C, 3 months)	50	47.81 ± 2.47	5.17	-4.38
	80	84.54 ± 2.78	3.29	5.62
	1500	1337.55 ± 12.59	0.94	-10.83
	4000	3655.47 ± 13.88	0.38	-8.61

3.6 Conclusions

The developed reversed phase HPLC methods were selective, sensitive, precise, accurate and robust. These methods involve simple procedure with high throughput. The analytical method was successfully utilized for the quantification of donepezil in the in-house developed nano-formulations, *in-vitro* drug release kinetics and cellular uptake studies. Moreover, the advantages of the developed bio-analytical method include simple sample preparation procedure, free from matrix interference, requirement of low plasma sample volume (200 µL) and a short total run time (06 min). This method was used for pharmacokinetic and biodistribution studies of pure drug and developed nano-formulations.

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