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# Development And Validation of Novel LC-MS Method For Quantification Of Donepezil From Human plasma.

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**Abstract:** Donepezil is used worldwide for the treatment of Alzheimer's disease, where it is used to increase cortical acetylcholine. We present a simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed and validated for the quantification of Donepezil in Human Plasma. Quetiapine was used as the internal standard. The simple and cost effective Liquid-Liquid Extraction method is applied for the extraction of Donepezil and Internal standard from Plasma. A rapid isocratic separation of Donepezil is achieved by a short C18 column using mobile phase of Acetonitrile and 1mM Ammonium acetate buffer (92:8 v/v, (%) ) at flow rate of 0.5 ml/min. The run time is 2.5 minutes suggests high throughput of the proposed method. The collision-induced transition *m/*z 380 → 91 was used to analyze donepezil in selected reaction monitoring mode. The signal intensity of this transition was found to relate linearly with donepezil concentrations in plasma from 0.1- 42 ng/ml. The lower limit of quantification of the LC-MS/MS method was 0.1ng/ml. The intra-and inter-day precisions were below 10.0% and the accuracy was between + 5.0% and - 5.0%. The validated LC/MS/MS method was applied to pharmacokinetic study on healthy male volunteer who received single oral dose of 10 mg Donepezil hydrochloride. The maximum plasma concentration observed was 17.05ng/ml at 2.5 hours post dosing. This validated LC-MS/MS is rapid, sensitive, specific and cost-effective method for determining donepezil in human plasma samples.
**Keywords:** Donepezil, LC-MS/MS study, Bioequivalence, Development and validation.

## INTRODUCTION

Donepezil (DNP) is chemically (RS)-2-[(1-benzyl-4-piperidyl)methyl]- 5,6-dimethoxy-2,3-dihydroinden-1-one and it's molecular weight and molecular formula is 379.49 and $C_{24}H_{29}NO_3$ respectively. The Hydrochloride salt of Donepezil is used for the treatment of Alzheimer's disease. Donepezil hydrochloride is a white crystalline powder and is freely soluble in chloroform, soluble in water and in glacial acetic acid, slightly soluble in ethanol and in acetonitrile and practically insoluble in ethyl acetate and in n-hexane. It is melting at about 206°C. The acid dissociation constant of DNP is 8.8. The partition coefficient of DNP in octanol/water system is Log P = 3.6[1].

DNP is well absorbed with a relative oral bioavailability of 100% and reaches peak plasma concentrations in 3 to 4 hours. Pharmacokinetics are linear over a dose range of 1-10 mg given once daily. Following multiple dose administration, DNP accumulates in plasma by 4-7 fold and steady state is reached within 15 days. According to one study there was a statistically significant positive correlation between plasma concentrations of donepezil and acetylcholinesterase inhibition; the EC50 (50% effect)

was obtained at a concentration of 15.6 ng/mL and a plateau of inhibition (80-90%) was achieved at a concentration greater than 50 ng/mL. The elimination half-life of donepezil is approximately 70 hours hence it can be taken once a day. DNP is approximately 96% bound to human plasma proteins, mainly to albumins (about 75%) and alpha1 - acid glycoprotein (about 21%) over the concentration range of 2 - 1000 ng/ml. DNP is both excreted in the urine intact and extensively metabolized to four major metabolites, two of which are known to be active, and a number of minor metabolites, not all of which have been identified. DNP is metabolized by the CYP 450 isoenzymes 2D6 and 3A4 and undergoes glucuronidation[2].

Previous investigations have shown that DNP in biological fluids can be detected using high-performance liquid chromatography (HPLC) equipped with a ultraviolet spectrometric (UV)[3–9, 17], fluorescence (FL)[10, 11] or mass spectrometric (MS)[12–13] detector as well as can also be analysed by Capillary electrophoresis[16, 17]. The HPLC/UV method has been used to determine DNP in human plasma[9, 10, 12, 14] but the method is insensitive (1.0 ng/mL) and requires a large volume of blood sample (1.0mL)[3–9]. Radwan et al.[9] have reported a stereoselective HPLC/UV assay for determining donepezil enantiomers. The assay has a low limit of quantification (LLOQ) of about 50 ng/mL and requires at least 20min to complete the run. LC/MS[12-13] also has been used to analyze DNP in different biological fluids. The LLOQ of LC/MS is about 0.1ng/mL and the run time for each sample is longer than 7min. A sensitive and enantioselective method has been developed on LC-MS/MS[16] using a deuterium-labelled internal standard (IS) the LLOQ is 0.0206ng/mL and on HPLC with UV spectrometer[18], for estimation of DNP enantiomers in human plasma.

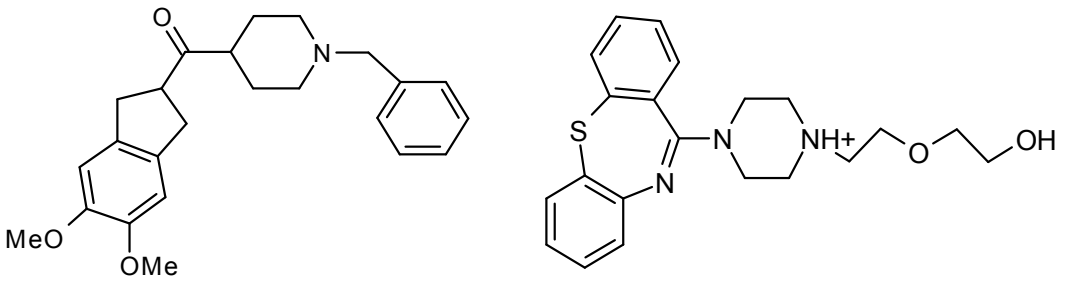
In this paper we describe a rapid and simple LC-MS/MS method for determination of DNP in human plasma using LC-MS/MS detection that allows rapid processing of large number of plasma samples. The method employed a Liquid-Liquid extraction procedure for the extraction of the drugs that reduces the cost of analysis. This bioanalytical method is being validated as per the FDA guidelines[19].

## EXPERIMENTAL

### Chemical and reagents

Working standards of Donepezil Hydrochloride of purity 99.5%, on as is basis and Quetiapine Fumarate of purity 98.6%, on as is basis were kindly gifted by Unichem Laboratories Ltd. (Mumbai, India). The chemical structure of Donepezil and Quetiapine is given in Figure 1 (A) and (B). Acetonitrile and Methanol was obtained from J. T. baker (Mumbai, India); ammonium acetate was supplied by Merck, (Mumbai, India). AnalR grade dichloromethane and t-butyl methyl ether was supplied by Qualigen's fine chemicals (Mumbai, India). High purity water was obtained from Millipore, Milli-Q (Milford, MA, USA) water purification system. Blank human plasma was obtained from the blood bank (Mumbai, India) and stored at -50°C prior to use.

**Figure 1 : Molecular structure of (A) Donepezil; (B) Quetiapine**




(A) (B)

**Instrumentation:**
The liquid chromatographic system consist of LC-Shimadzu LC10 from Shimadzu, an auto sampler of Shimadzu (SIL-HTc) coupled with an applied Biosystems SCIEX a triple quadrupole mass spectrometer (API 4000), equipped with electrospray ionization (ESI), Date acquisition and processing were controlled by Applied Biosystems/MDS SCIEX Analyst software (version 1.4.2)

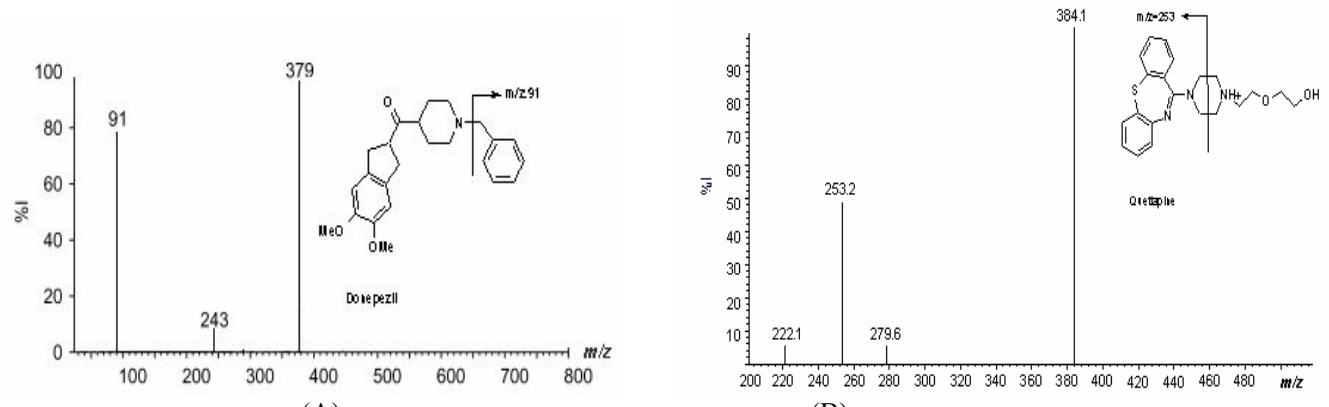
**Chromatographic condition :**
Chromatographic separation was achieved using Waters Symmetry C18 (50 x 4.6) mm, 3.5 µm column thermostated at 30°C. The mobile phase was an Acetonitrile and 1mM Ammonium acetate buffer (94:6 v/v, (%)). The separation was performed under isocratic conditions with a constant flow rate of 0.5 ml/min. The injection volume was 10µl. The run time is 2.5 minutes, with approximate retention time of 1.62 min for DNP and 1.82 min for QTP.

**Mass spectrometric condition:**
The mass spectrometer was operated in the positive ion mode. Quantification was performed using selected reaction monitoring (SRM) for the transitions of m/z 380 → 91 for DNP and m/z 384 → 253 for QTP (IS), respectively with a scan time of 0.3 s per transition. The tuning parameters were optimized for DNP and IS by infusing a solution, containing 1 mg/mL of each analyte, at a flow rate of 10 mL/min into the mobile phase (0.5 mL/min) using a post-column 'T' connection. The optimized collision energies chosen for donepezil and IS were 44 and 31eV, respectively. Figure 2 shows the product ion mass spectra of the $[M+H]^+$ ion of donepezil and IS.

**Preparation of stock solutions**
Stock solution containing 1 mg/ml of DNP was prepared in methanol. While 2.5µg/ml of QTP was prepared in diluent solvent (acetonitrile : milli-Q water (90:10)).

**Calibration Curve standard and Quality control sample**
Standard curves based on peak-area ratios (drug to IS) were prepared by spiking drug-free plasma with standard solution of DNP of 0.10 ng/mL to 42.00 ng/mL. Prepared quality control samples consisted of 0.11ng/mL (LLOQC), 0.31 ng/mL (LQC), 17.19 ng/mL (MQC) and 30.16 ng/mL (HQC). These samples were stored below -50°C until used, Six sets of LQC and HQC were transferred to below -50°C freezer for generation of -50°C stability.

**Figure 2: Product ion mass spectra of [M+Z]+ ions of (A) Donepezil and (B) Quetiapine (internal standard)**




(A) (B)

**Sample preparation and extraction procedure :**

The spiked plasma samples were retrieved from the deep freezer and thawed. The thawed samples were vortexed to ensure complete mixing of the contents. A 300 µL of the samples was pipetted into a test tube. 25 µL of 2.5 µg/mL quetiapine dilution was added to it as an internal standard (IS), except in blank plasma samples and votexed.

To a test tube containing 300 µL of sample 4mL of extraction solution t-Butyl Methyl Ether : Dichloromethane (TBME:DCM) :: 80:20, v/v was added. This sample is reciprocated at 100-200 RPM for 20 minutes followed by centrifugation at 2000 RPM for about 5 minutes at 4°C to 6°C. Further 3 mL of supernatant is evaporated to dryness at 40°C in nitrogen evaporator. The dried sample obtained was reconstituted in 400 µL of mobile phase and transferred into HPLC vial for analysis.

## VALIDATION

Assay performance was evaluated through determination of specificity, linearity, quantification limit, precision, accuracy, recovery and stability.

Specificity was investigated by analyzing six drug-free bottled plasma and volunteer samples for interference of endogenous compounds. The standard curve was obtained through analysis of calibration plot of peak area versus the corresponding drug concentrations. Linearity of the standard curve was evaluated using least-squares linear regression analysis with weighting $1/ x^2$. Quantification limit was defined as the lowest drug concentration on the calibration curve. Intra-and inter-day precision were determined by repeated analysis of quality control plasma samples on the same day and on different days. Five replicates of each of the three quality control standards and the lowest calibrator were analysed in a batch to establish the method's precision (% relative standard deviation was calculated). Recovery was evaluated by comparing the observed concentration with the true value of spiked controls. The recoveries of DNP and QTP were determined by comparing the response of quality control plasma samples with the response of identical standards prepared in the mobile phase which did not undergo sample pre-treatment. The recovery was evaluated at all the three quality control concentration levels as well at LLOQC. Stability of DNP in plasma samples stored at -50°C, in plasma samples after freeze-thaw cycles and in pre-treated plasma samples (short term) was evaluated. Stability of DNP and QTP were also evaluated in respective stock solution and in mobile phase in the processed samples. Higher and lower quality control standards were used for evaluating the stability of spiked plasma samples subjected to various conditions and the results compared with initial readings. To evaluate the freeze-thaw stability, plasma controls were subjected to three cycles of freezing and thawing. Short-term or temperature stability of spiked plasma solutions was evaluated by leaving the samples at room temperature for 6 hours. The processed samples were reconstituted with mobile phase and left in the autosampler at 4°C for 101hours to find the post-processing stability.

## RESULT AND DISCUSSION

**Chromatography:**

Representative chromatograms of drug free blank plasma, blank plasma with 16.5 ng/ml of Donepezil and 156 ng/ml of Quetiapine, LLOQC sample, Volunteer's 4 hours sample after an oral dose of 10mg Donepezil hydrochloride are given Figure 3 (A), (B), (C) and (D) respectively.

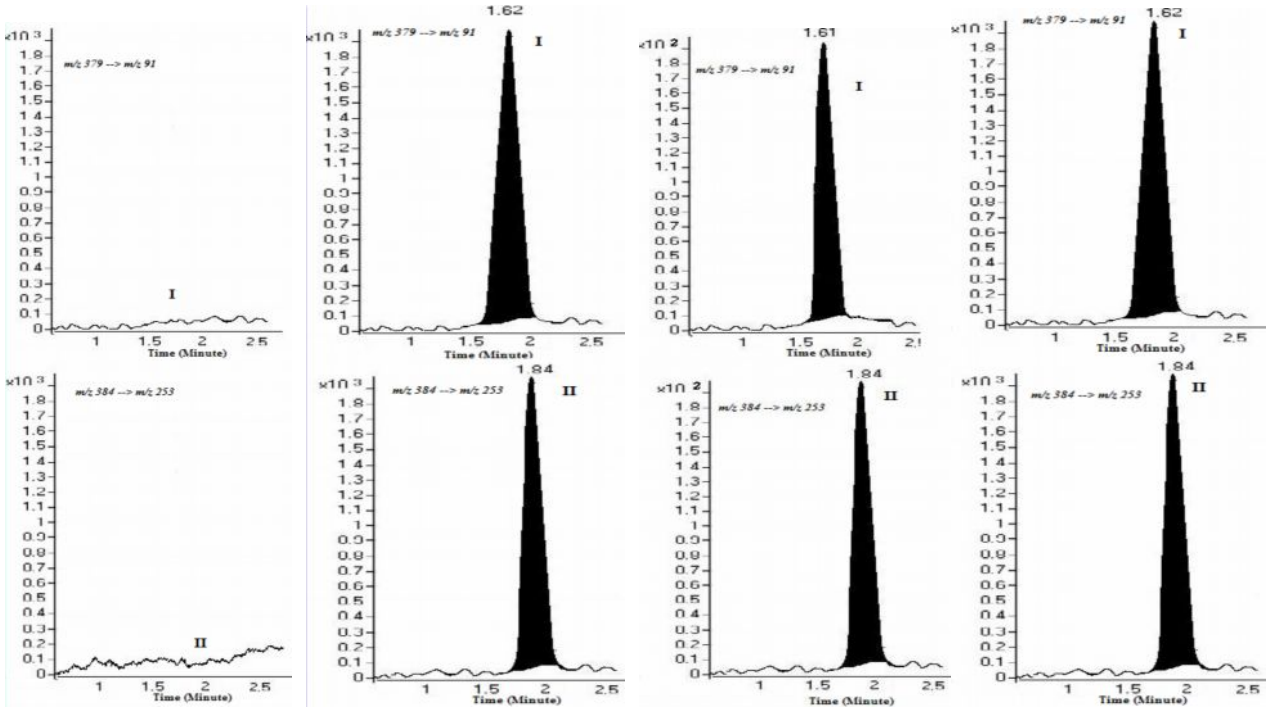
**Selectivity and sensitivity:**

Six different lots of plasma were harvested under controlled conditions in a fasted state, extracted and analyzed using the developed method. No interferences were observed at the retention times of DNP and QTP. Samples were compared with those obtained from an extract of a previously spiked plasma sample at LLOQ containing DNP and QTP. Representative chromatograms of the blank human plasma and blank human plasma spiked with DNP and QTP are given in Fig 3 (A) and (B) respectively.

The effect of potentially interfering drugs (PID) (caffeine, nimesulide, paracetamol, ibuprofen and aspirin) on DNP analysis was performed by spiking PIDs at their approximately $C_{max}$ concentration in the MQC sample (16.090 ng/mL) in triplicate. Samples were prepared by spiking 25µL of each interfering drug in the MQC containing QTP (300 µL MQC + 25 µL QTP). Similarly blank samples also prepared by taking 300 µL of blank plasma, 25 µL of the diluent solution and 25 µL of potentially interfering drug. The samples were quantified against calibration curve. The back calculated concentrations of MQC sample spiked with PID were found to be within $\pm$15 % of the actual concentration of the MQC sample. Hence the above mentioned PIDs have no effect on the DNP analysis.

The lowest limit of quantification for donepezil was set at the concentration of the LLOQ 0.100ng/mL. The precision and accuracy for donepezil at this concentration was found to be 2.02% and 96.17% respectively.

**Figure 3: Representative chromatogram of (A) Drug free plasma; (B) Plasma spiked with 16.5 ng/ml of Donepezil and 156 ng/ml of Quetiapine; (C) LLOQC sample; (D) Volunteer's 2 hours sample after and oral dose of 10mg Donepezil hydrochloride.**




(A) (B) (C) (D)

**I : Donepezil II: Quetiapine.**

**Goodness of fit:**

The data of five precision and accuracy batches was subjected of goodness of fit analysis after taking the back calculated concentration of CC standards meeting the acceptance criterion using 1/x and $1/x^2$ weighing. After performing the goodness of fit, $1/ x^2$ was found to be the best fit for regression.

**Linearity**

A regression equation with a weighting factor of $1/ x^2$ of DNP to QTP concentration was judged to produce the best fit for the concentration-detector response relationship for DNP in human plasma. Correlation coefficient ($r^2$) was greater than 0.99 in the concentration range of 0.1ng/mL to 42.92ng/mL, for DNP.

**Precision and accuracy :**

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQC, LQC, MQC and HQC samples respectively during the course of validation. The intra-day and inter-day precisions were less than 10.0% for each QC level of DNP. The accuracy, determined from QC samples, was within +5.0% for each QC level. The results obtained are given in Table 1.

**Table 1 : Result for Precision and Accuracy.**

| | Inter-day precision | | Intra-day Precision | |
|---|---|---|---|---|
| Level | % Accuracy | % CV | % Accuracy | % CV |
| LLOQC | 100.94 | 9.80 | 96.58 | 9.44 |
| LQC | 102.54 | 6.15 | 102.46 | 4.26 |
| MQC | 98.02 | 4.87 | 96.29 | 3.68 |
| HQC | 101.66 | 6.15 | 100.12 | 4.61 |

CV=Coefficient of Variation.

**Recovery:**

Prepared 6 sets of recovery comparison samples by spiking dilution of quality control samples (LQC, MQC, HQC) of DNP, 125 µL of QTP and 1800 µL of reconstituting solution, representing 100% extraction and injected. The recovery comparison samples of donepezil were compared against extracted samples of LQC, MQC and HQC. The mean overall recovery of donepezil was 99.82% with precision of 6.12%. The mean recovery of internal standard was 96.57%.

**Stabilities:**

***Refrigerated standard stock solution stability:***

Refrigerated stock solution stability was carried out for 9 days by making six injections of stability standard stock solution and the fresh standard (comparison stock) stock solution of DNP sample concentration. The response of stability sample was corrected by multiplying with correction factor. The % change for DNP was 3.70 and % stability was 96.30%. The refrigerated stability of QTP was carried out for 9 days and the % change was 1.17% and % stability was 101.17%.

Similarly short term standard stock solution stability was carried out for 6 and 24 hours at room temperature. The stability of standard stock solution at 6 and 24 hours for DNP was 98.19% and 96.33% respectively and for QTP was 98.50% and 98.68% respectively.

**Correction factor** was calculated as follows:

$$\text{Correction factor} = \frac{\text{Corrected concentration of comparison (fresh) standard stock solution}}{\text{Corrected concentration of stability standard stock solution}}$$

Note : Corrected concentration : Concentration corrected using potency, actual amount weighed and molecular weight of the compound.

$$\% \text{ Stability} = \frac{\text{Mean corrected response of stability stock}}{\text{Mean responses of comparison stock}} \times 100$$

***Standard stock solution stability at room***

Standard stock solution stability of analyte and QTP was carried out for 6 hours at room temperature by injecting each six replicates of stock dilutions of stability standard stock solution and the fresh standard (comparison stock) stock solution.

The response of stability sample was corrected by multiplying with correction factor. The standard sock solution % stability for DNP was 96.19%, and for QTP was 98.50%.

***Short-Term room temperature stock dilution stability***

Room temperature stability of stock solution was carried out for 24 hours by injecting six injections of prepared stock dilutions of DNP sample concentration kept at room temperature against the dilution kept in refrigerator. The percentage change for DNP was 1.67% and for QTP was 1.32% respectively.

***Auto sampler stability :***

Six sets of QC samples at LQC and HQC were processed and placed in the auto sampler. These samples were injected after a period of around 101 hours and were quantified against freshly spiked calibration curves standards. The samples were found to be stable over a period of 101 hours. The percentage nominal at around 101 hours ranged form 98.64% (HQC) to 102.15% (LQC) and precision ranged form 0.73% (HQC) to 2.58% (LQC) respectively.

***Freeze-Thaw stability :***

The stability in human plasma was determined for three freeze-thaw cycles. Six injections of LQC and HQC were analyzed after undergoing three freeze-thaw cycles. The freeze-thaw quality control samples

were quantified against the freshly spiked calibration curve standards. The freeze thaw cycles were carried out in duplicate. The percent nominal ranged from 99.492% (HQC) to 106.452% (LQC) and precision ranged from 1.09% (HQC) to 1.92% (LQC) respectively were obtained.

***Short-Term stability of plasma sample below -50°C***

The stability of DNP for temporary storage of plasma samples below -50°C was studied for 10days by quantifying six sets each of LQC and HQC against the freshly spiked standards. The mean % nominal ranged from 97.21% (LQC) to 101.58% (HQC) and the precision ranged from 1.03% (HQC) to 5.54% (LQC) for 10days.

***Bench top stability :***

Short-term room temperature stability was determined around 8 hours, using six sets each of LQC and HQC. The quality control samples were quantified against the freshly spiked calibration curve standards.
Donepezil was found to be stable around 8 hours. The percent nominal ranged from 97.12% (HQC) to 100.54% (LQC) and the precision ranged from 0.70% (HQC) to 2.42% (LQC).

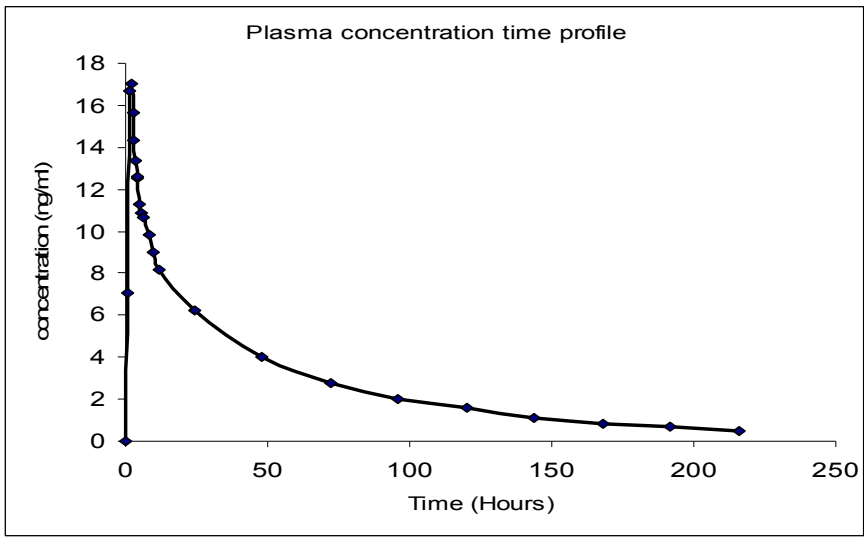
**APPLICATION: PHARMACOKINETIC STUDY**

The proposed validated bioanalytical method was successfully applied to determine DNP concentration in plasma for pharmacokinetic study in healthy male volunteer, who was orally, administered a tablet containing 10mg Donepezil Hydrochloride in a fasted state. Venous blood samples collected into heparinised tubes at the following time points: immediately before dose administration (0.00) and at, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 8.00, 10.00, 12.00, 24.00, 48.00, 72.00, 96.00, 120.00, 144.00, 168.00, 192.00 and 216.00 hours after dosing. Blood samples were centrifuged (2000rpm for 10min); plasma was separated and stored at -50°C until assay. High-throughput sample analysis is of particular importance for studies that require the analysis of large numbers of samples, and the described Liquid-Liquid Extraction method of sample preparation is suitable for this purpose. The method was sensitive enough to monitor DNP plasma concentration up to 216 hours. The maximum plasma concentration was 17.05ng/ml which occurred at about 2.5 hours post dosing. Figure 4 shows the mean plasma concentration-time curve for the DNP.

**CONCLUSION:**

The objective of this work was to develop a simple, cost effective rugged and a high throughput method for estimation of DNP in human plasma. The method consists of a simple sample pre treatment by liquid-liquid extraction to give consistent and reproducible recoveries of DNP. The run time is 2.5 minutes suggests high throughput of the proposed method. The maximum on-column loading of DNP was only 0.42ng/ml per injection volume of 10µl for DNP. This was considerably less compared to the reported procedure[3-9], which helps in maintaining the efficiency and the lifetime of the column. Moreover, the limit of quantification is low enough to monitor DNP concentration in pharmacokinetic study with good intra and inter-assay reproducibility (%CV) for use in quality controls.

**Figure 4: Mean Plasma concentration-time profile of donepezil after an oral dose of 10mg Donepezil hydrochloride to healthy volunteer**.



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