



CODEN [USA]: IAJPBB

ISSN: 2349-7750

**INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES**<http://doi.org/10.5281/zenodo.1253713>Available online at: <http://www.iajps.com>**Research Article****METHOD DEVELOPMENT AND VALIDATION OF
DONEPEZIL HYDROCHLORIDE BY RP-HPLC****Sharmila Dusia^{a,*}, Ramanamma.L, Prasad.DSVS**Pharmaceutical Sciences, Andhra University, Visakhapatnam-530017 ,
Andhra Pradesh**Abstract:**

A RP-HPLC for Donepezil hydrochloride in tablets was developed and validated. Donepezil hydrochloride is a reversible inhibitor of acetylcholinesterase, indicated for the treatment of mild to moderate dementia of the Alzheimer's type. The HPLC method was performed with a unisol reversed phase C18 column (150×4.6 mm, 3 μm) detection at 268 nm and a mixture of Acetonitrile: Water (50:50) as mobile phase. Typical retention time for Donepezil was 4.407 mins. The method was statistically validated for linearity, accuracy, precision and selectivity following ICH recommendations. Due to its simplicity and accuracy, the method can be used for routine quality control analysis.

Keywords: *Donepezil hydrochloride; Assay; HPLC; Validation.***Corresponding author:****Sharmila Dusia^{a,*},**
Pharmaceutical Sciences,
Andhra University,
Visakhapatnam-530017 ,
Andhra Pradesh

QR code



Please cite this article in press Sharmila Dusia et al., Method Development and Validation of Donepezil Hydrochloride by RP-HPLC, Indo Am. J. P. Sci, 2018; 05(05).

INTRODUCTION:

Analytical chemistry is used to determine the qualitative and quantitative composition of material under study. Both these aspects are necessary to understand the sample material. Analytical chemistry is divided into two branches quantitative and qualitative. A qualitative analysis gives us the information about the nature of sample by knowing

about the presence or absence of certain components. A quantitative analysis provides numerical information as to the relative amount of one or more of this component. For analyzing the drug samples in bulk, pharmaceutical formulations and biological fluids, different analytical methods are routinely being used.

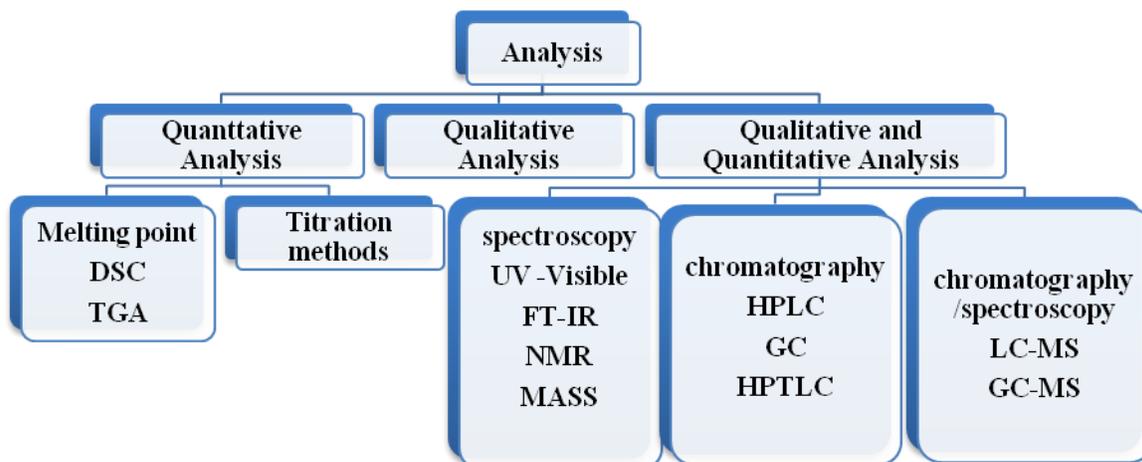


Fig.1 Qualitative and Quantitative Analysis

Ref: Phani.R.S.Ch, K.R.S.Prasad: Scientific approach for RP-HPLC method development: complete review; International journal of science innovations and discoveries, 2(6), 2012, 218-228.

In non-instrumental, the physic chemical properties are use to analyze the sample. The instrumental methods of analysis are based upon the measurements of some physical property of substance using instrument to determine its chemical composition. The instrumental methods are simple, precise and reproducible as compared to classical methods. Therefore, analytical methods developed using sophisticated instruments such as spectrophotometer, HPLC, GC and HPTLC have wide applications in assuring the quality and quantity of raw materials and finished products(1).

CHROMATOGRAPHY

Chromatography is a technique used for separation of the components of mixture by continuous distribution of the component between two phases. One phase moves (mobile phase) over the other phase (stationary phase) in a continuous manner. Chromatography according to USP can be defined as a procedure by which solute are separated by a differential migration process in a system consisting of two or more phases, one of which move continuously in a given direction.

PRINCIPLE OF CHROMATOGRAPHY**Adsorption Chromatography:**

When the stationary phase is a solid and mobile phase is liquid or gaseous phase, it is called Adsorption Chromatography. The different solutes travelled different distances through the solid, carried along by the solvent. Each solute has its own equilibrium between adsorption onto the surface of the solid and solubility in the solvent, the least soluble or best adsorbed ones travel more slowly. The result is a separation into bands containing different solutes.

Examples: Thin layer chromatography, Column chromatography, Gas-solid chromatography.

Partition Chromatography:

When the stationary phase and mobile phase are liquid, it is called Partition Chromatography. The solutes distribute themselves between the moving and the stationary phases, with the more soluble component in the mobile phase reaching the end of the chromatography column first.

Example: Paper partition chromatography, Gas-liquid chromatography.

THEORY OF CHROMATOGRAPHY

Two theoretical approaches have been developed to describe the processes involved in the passage of solutes through a chromatographic system.

The plate theory:

According to Martin and Synge, a chromatographic system consists of discrete layers of theoretical plates. At each of these, equilibration of the solute between the mobile and stationary phases occurs. The movement of solute is considered as a series of stepwise transfers from plate to plate.

The rate theory:

This theory considers the dynamics of the solute particles as it passes through the void space between the stationary phase particles in the system as well its kinetic as it is transferred to and from the stationary phase.

TYPES OF CHROMATOGRAPHY**Normal Phase Chromatography:**

In Normal Phase mode the stationary phase is polar and the mobile phase is non polar in nature. In this technique, non polar compounds travel faster and are eluted first. This is because of the lower affinity between the non polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reversed Phase Chromatography:

It is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. The polar compound gets eluted first in this mode and non polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C18, C8, C4, (in the order of increasing polarity of the stationary phase). An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity.

Ion Exchange Chromatography:

The stationary phase contains ionic groups like NR_3^+ , SO_3^- -which interact with the ionic groups of

the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Ion Pair Chromatography:

This technique is also referred to as Reversed Phase Ion Pair Chromatography or Soap Chromatography. It may be used for the separation of ionic compounds and this method can also substitute for Ion Exchange Chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulombic association species formed between two ions of opposite electric charge) with suitable counter ions.

Affinity Chromatography:

This technique uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Size Exclusion Chromatography:

It separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

METHOD DEVELOPMENT (2-4):

Analytical method development and validation play important roles in the drug discovery and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of drug products. There are many factors to consider when developing methods. The initially collect the information about the analyte's physiochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis. The majority of the analytical development effort goes into validating a stability indicating HPLC method. The goal of the HPLC-method is to separate and quantify the main active drug, any reaction impurities, all available synthetic intermediates and any degradants.

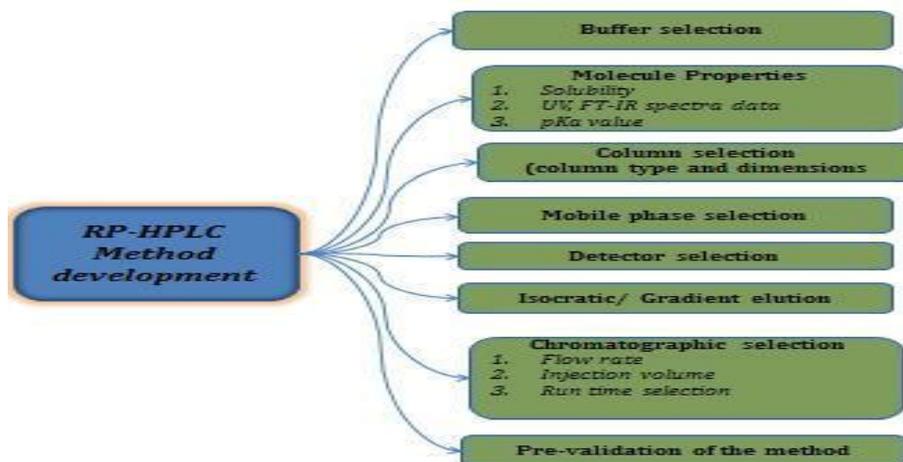


Fig.2 HPLC method development approach

Ref: Phani.R.S.Ch, K.R.S.Prasad: Scientific approach for RP-HPLC method development: complete review; International journal of science innovations and discoveries, 2(6), 2012, 218-228.

There are many steps involve in method development which are:

- Physicochemical properties of drug.
- Set up HPLC conditions.
- Sample preparation.
- Method optimization.
- Validation of developed method.

Physicochemical properties of drug:

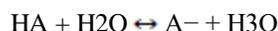
For method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. In a non-polar covalent bond, the electrons are shared equally between two atoms. A polar covalent bond is one in which one atom has a greater attraction for the electrons than the other atom. The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and non-polar, e.g. benzene.

Selection of diluents is based on the solubility of analyte. The analyte must be soluble in the diluents and must not react with any of the diluents components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components. pH and pKa plays an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion,

$$\text{pH} = -\log_{10} [\text{H}_3\text{O}^+]$$

The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for

ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. The acidity of an aqueous solution is determined by the concentration of $[\text{H}_3\text{O}^+]$ ions. Thus, the pH of a solution indicates the concentration of hydrogen ions in the solution. The concentration of hydrogen ions can be indicated as $[\text{H}^+]$ or its solvated form in as $[\text{H}_3\text{O}^+]$ whose value normally lies between 0 and 14. The lower the pH, the more acidic is the solution. The pH of a solution can be changed simply by adding acid or base to the solution. The pKa is characteristic of a particular compound, and it tells how readily the compound gives up a proton. An acid dissociation constant is a particular example of equilibrium constant. For the specific equilibrium between a monoprotic acid, HA and its conjugate base A,



The position of equilibrium is measured by the equilibrium constant, K_{eq} .

$$K_{\text{eq}} = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{H}_2\text{O}][\text{HA}]}$$

Now in dilute solutions of acid, $[\text{H}_2\text{O}]$ stays roughly constant. Therefore define a new equilibrium constant the acidity constant K_{a} .

$$K_{\text{a}} = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{HA}]}$$

This is also in logarithmic form are follows: $\text{pK}_{\text{a}} = -\log_{10} K_{\text{a}}$.

It turns that the pKa of an acid is the pH at which it is exactly half dissociated. This can be shown by rearranging the expression for K_{a} :

$$\text{pH} = \text{pK}_{\text{a}} - \log\left(\frac{[\text{AH}]}{[\text{A}^-]}\right)$$

At half-neutralization $[A^-] / [HA] = 1$; since $\log(1) = 0$, the pH at half-neutralization is numerically equal to pKa. Conversely, when $\text{pH} = \text{pKa}$, the concentration of HA is equal to the concentration of A^- . The buffer region extends over the approximate range $\text{pKa} \pm 2$, though buffering is weak outside the range $\text{pKa} \pm 1$. At $\text{pKa} \pm 1$, $[A^-]/[HA] = 10$ or $1/10$. If the pH is known, the ratio may be calculated. This ratio is independent of the analytical concentration of the acid. When the pKa and analytical concentration of the acid are known, the extent of dissociation and pH of a solution of a monoprotic acid can be easily calculated (5-9).

Set up HPLC conditions:

A buffer is a partially neutralised acid which resists changes in pH. Salts such as Sodium Citrate or Sodium Lactate are normally used to partially neutralise the acid. Buffering Capacity is the ability of the buffer to resist changes in pH:

- Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases

Table.1 properties of some common buffers used for reversed phase HPC

Buffer	pKa	Useful pH range
Ammonium acetate	4.8	3.8-5.8
	9.2	8.2-10.2
Ammonium formate	3.8	2.8-4.8
	9.2	8.2-10.2
KH ₂ PO ₄	2.1	1.1-3.1
KH ₂ PO ₄ /K ₂ PO ₄	7.2	6.2-8.2
Potassium acetate/acetic acid	4.8	3.8-5.8
Borate	9.2	8.2-10.2
Ammonium hydroxide/ammonia	9.2	8.2-10.2
Trifluoro acetic acid	<2	1.5-2.5
Potassium formate/formic acid	3.8	2.8-4.8

General considerations for buffer selection:

- Phosphate is more soluble in methanol/water than in acetonitrile/water or Tetrahydrofuran/water.
- Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
- Ammonium salts are generally more soluble in organic/water mobile phases.
- TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.

- The closer the buffered pH is to the pKa, the greater the Buffering Capacity
- Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0.

Consideration of the affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection are important in reversed-phase chromatography (RPC) method development of ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds (10-12).

Buffer selection: Choice of buffer is typically governed by the desired pH. The typical pH range for reversed-phase on silica-based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value < 2 units of the desired mobile phase pH.

- At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
- Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 to 48 hours. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
- After buffers are prepared, they should be filtered through a 0.2- μm filter.
- Mobile phases should be degassed.

Buffer concentration:

Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This

will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Phosphate buffers can be replaced with sulfate buffers when analyzing organophosphate compounds(13).

Selection of detector:

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability and/or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis.

Photodiode Array (PDA) Detector offers advanced optical detection for analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds (14-15).

Column selection:

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution.

There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased separation efficiency.

However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged. In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar, causing polar peaks to generally elute earlier than non-polar peaks. To create a stationary phase for reverse phase chromatography on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface.

Due to steric constraints, only about 1/3 of the surface silanols are derivatized. The remaining free silanols can interact with analytes, causing peak tailing. Typically, after the derivatization of a column with the desired stationary phase, the column is further reacted with chloro trimethylsilane to end cap the remaining free silanols and improve the column efficiency. Common stationary phases are C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes(16-19). Commonly used reverse phase columns and their uses are listed below.

Table.2 List of commonly used reversed phase columns

Column type	Uses	Example
C3(propyl), C4(butyl), C5(pentyl)	Ion pairing chromatography	Zorbax SB-C3 YMC-pack-C4 Luna C5
C8(octyl)	For non polar analytes	Zorbax SB-C8 YMC-pack-MOS Luna C8
C18(octadecyl)	For non polar analytes	Zorbax SB-C18 Zorbax Extend C18
Phenyl(phenyl propyl)	For aromatic compound separation	Zorbax SB-phenyl Luna phenyl
Nitrile(cyanopropyl)	For polar analytes	Zorbax SB-CN Luna CN

Column temperature:

Column temperature control is important for long-term method reproducibility as temperature can affect selectivity. A target temperature in the range of 30–40 °C is normally sufficient for good reproducibility. Use of elevated temperature can be advantageous for several reasons. First, operating at a temperature higher than ambient reduces the viscosity of the mobile phase and thus the overall backpressure on the column. Lower system pressures allow for faster flow rates and thus faster analyses. The temperature may also affect selectivity patterns because analytes will respond dissimilarly to different temperatures.

Finally, use of a column oven eliminates variability due to normal fluctuations in the air temperature surrounding the column. While temperature is a variable that can affect selectivity ' α ' its effect is relatively small. Also, the k' generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. Some effect when there is a significant difference in shape and size. Overall, it is better to use solvent strength to control selectivity than to use temperature; its effect is much more dramatic. An increase of 1°C will decrease the k' by 1 to 2%, a both ionic and neutral samples are reported to show significant changes in a with temperature changes. Possible temperature fluctuations during method development and validation, it is recommended that the column be thermo stated to control the temperature.

MOBILE PHASE**Solvent type**

Solvent type (methanol, acetonitrile, and tetrahydrofuran) will affect selectivity. The choice between methanol and acetonitrile may be dependent on the solubility of the analyte as well as the buffer used. Tetrahydrofuran is least polar among these three solvent, often responsible for large changes in selectivity and is also incompatible with the low wavelength detection required for most pharmaceutical compounds.

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the drug molecule. Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture

respectively. The aqueous buffer serves several purposes.

At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated when ionized the analyte will elute more quickly but with improved peak shape. Acidic analytes in buffers of sufficiently low pH will remain unchanged, increasing retention. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pKa of a compound is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non ionizable sample components. Typically a 10-50 mM solution of an aqueous buffer is used. The most commonly used aqueous phase is H₃PO₄ in water i.e. phosphate buffer. The pH of a phosphate buffer is easily adjusted by using mono-, di-, or tribasic phosphate salts.

However, when phosphate salts are used the solution should be filtered to remove insoluble particles with 0.22µm filter paper. Other non-UV active acids and bases may also be used to effect differences in peak shape and retention.

pH of Mobile phase:

When the sample is eluted with a mobile phase with 100% organic there is no separation, as the sample is eluted in the void volume. This is because the sample is not retained; but retention is observed when the mobile phase solvent strength is decreased to allow equilibrium competition of the solute molecules between the bonded phase and the mobile phase.

When the separation is complex, many components are to be separated, and when the solvent strength is decreased and there is still no resolution between two close peaks, another organic solvent of a different polarity or even a mixture of two organics may need to be tried to effect separation. Additionally, mobile phase optimization can be enhanced in combination with bonded phase optimization (i.e., substituting C18 /C8 with cyano or phenyl). A goal for the band spacing of a solute (K') should be in the range of 4 to 9 and a run time of about 15 minutes or 20 minutes at most for most routine product release or stability runs.

SEPARATION TECHNIQUES**Isocratic separations:**

Isocratic, constant eluent composition means equilibrium conditions in the column and the actual

velocities of compounds moving through the column are constant. Analyte eluent and analyte-stationary phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak.

Gradient separation:

Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width varies depending on the rate of the eluent composition variation (gradient slope).

Changing Gradient elution is employed for complex multi component samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still attaining resolution of each component. This necessitates the implementation of a gradient.

Employing gradients shallow or steep allows for obtaining differences in the chromatographic selectivity. This would be attributed to the different slopes of the retention versus organic composition for each analyte in the mixture. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile phase conditions prior to the next sample injection and the start of the next gradient run.

Selection of isocratic or gradient mode depends on the number of active components to be resolved or separated. In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is to be performed, and the ratio between the total gradient time and the difference in gradient time between the first and last components are calculate. The calculated ratio is <0.25 , isocratic is adequate; when the ratio is >0.25 , gradient would be beneficial(20-21).

Sample preparation for method development:

The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered; the use of a 0.22 or 0.45 μm pore size filter is generally recommended for removal of particulates.

Filtration is a preventive maintenance tool for HPLC analysis. Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractable) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluents must be filtered to determine if a leachable component is coming from the syringe filter housing/filter(22).

METHOD OPTIMIZATION

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

METHOD VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The method validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines(23-29).

Validation parameters

The following are typical analytical performance characteristics which may be tested during methods validation:

1. System suitability determination.
2. Accuracy
3. Precision
4. Repeatability
5. Intermediate precision
6. Linearity
7. Detection limit

8. Quantitation limit
9. Specificity
10. Range
11. Robustness

SYSTEM SUITABILITY PARAMETERS:

System Suitability determination is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method. These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured

on a peak or peaks of known retention time and peak width.

System suitability parameters are used to determine the limits of the chromatographic system. System suitability (SST) parameters studied are as follows:

Resolution (Rs)

Resolution is the parameters describing the separation power of the complete chromatographic system relative to the particular components of the mixture. The resolution R_s two neighboring peak is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation the ideal value of R_s is 1.5. It is calculated by using the formula.

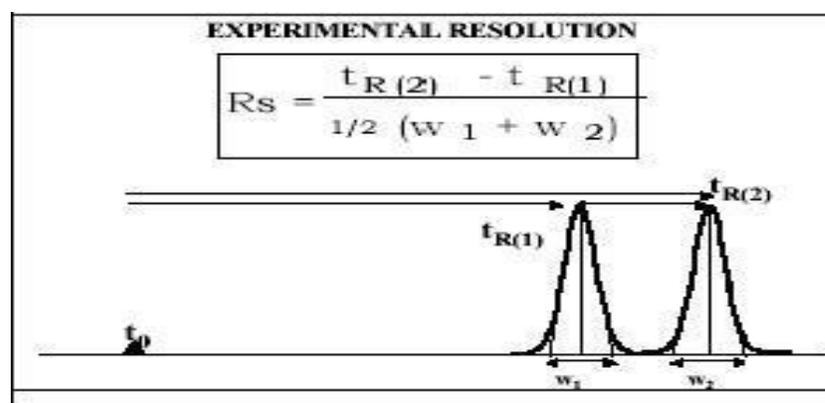


Fig.3 Resolution between two peak

Ref:

<http://www.chromforum.org/viewtopic.php?f=1&t=20079>.

Where, $t_{R(1)}$ and $t_{R(2)}$ are the retention times of components 1 and 2 and w_1 and w_2 are peak width of components 1 and 2.

Capacity factor (K')

Capacity factor, k' , is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column or TLC plate during an isocratic separation. The ideal value

of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$$k' = \frac{V_1 - V_0}{V_0}$$

Where, V_1 = retention volume at the apex of the peak (solute) and V_0 = void volume of the system.

Column efficiency (N)

It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 Plates/meter are ideal for a good system. Efficiency is calculated by using the formula:

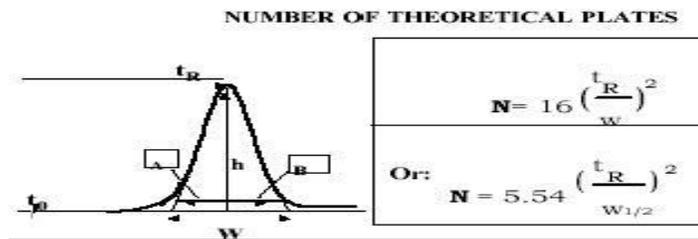


Fig.4 Number of theoretical plates

Ref: <http://infolinkads.com/Number-of-theoretical-plates.html>.

Where, t_R is the retention time and W is the peak width.

Peak asymmetry factor (As) and tailing factor:

Peak asymmetry factor (As) can be used as criteria of column performance. The peak asymmetry is measured at 10 % of full peak height, divided by corresponding front half width.

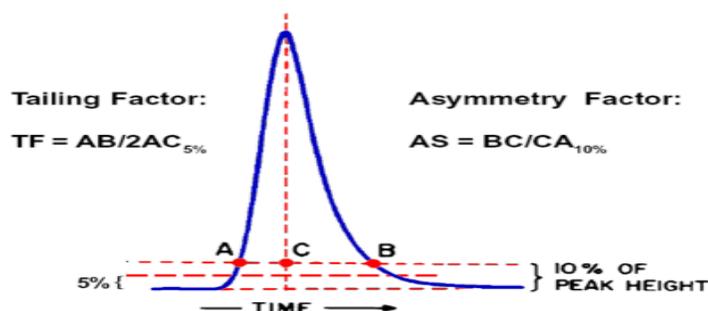


Fig.5 peak asymmetry

Ref: <http://www.chromforum.org/viewtopic.php?f=1&t=20079>.

Asymmetry factor is calculated by,

$$\text{Asymmetry factor} = BC/CA_{10\%}$$

B= Peak half width, A= Front half width

Good columns produce peaks with As values of 0.95 to 1 % (Exactly symmetrical peaks have an As value of 1.0%).

Peak purity:

Peak purity (or peak homogeneity) analysis of the main peak, is to assess for the presence of impurities under the main peak. It is an essential part of method validation.

when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method. It consists of two components: repeatability and intermediate precision

ACCURACY:

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte.

REPEATABILITY:

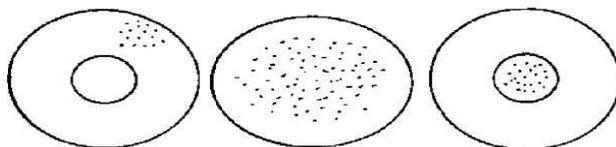
Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variations from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated. Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts. The precision is then expressed as the relative standard deviation.

PRECISION:

Precision of an analytical method is the degree of agreement among individual test results obtained

Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.

$$\%RSD = \frac{\text{std dev.} * 100}{\text{Mean}}$$



Precise not Accurate Accurate not Precise Accurate and Precise

LINEARITY:

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

DETECTION LIMIT:

The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.

The signal-to-noise ratio is determined by: $s = H/h$

Where H = height of the peak corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

QUANTITATION LIMIT:

The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytic al procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.

SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required.

RANGE:

Range is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical

procedure as a suitable level of precision, accuracy, and linearity.

ROBUSTNESS:

It is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage. Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.

Drug Name: Donepezil Hydrochloride

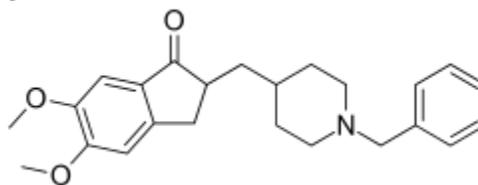
Drug Structure:

Fig.6 Structure of Donepezil



fig.7 Donepezil Tablets

Mechanism of action:

Donepezil is a piperidine derivative that is a centrally active, reversible inhibitor of acetylcholinesterase. This drug is structurally unrelated to other anticholinesterase agents. Donepezil's proposed mechanism of action involves the reversible inhibition of cholinesterases (eg. acetylcholinesterase), which prevents the hydrolysis of acetylcholine, and leads to an increased concentration of acetylcholine at cholinergic synapses. Evidence suggests that the anticholinesterase activity of donepezil is relatively specific for acetylcholinesterase in the brain.

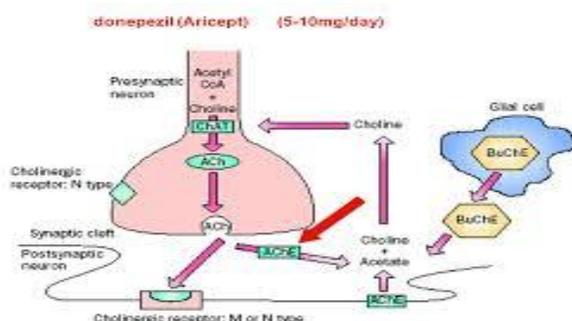


Fig.8 Mechanism of DONEPEZIL HCl

Pharmacokinetics (35): Pharmacokinetics of donepezil are linear over a dose range of 1-10 mg given once daily. The rate and extent of absorption of ARICEPT tablets are not influenced by food. Based on population pharmacokinetic analysis of plasma donepezil concentrations measured in patients with Alzheimer's disease, following oral dosing, peak plasma concentration is achieved for ARICEPT 23 mg tablets in approximately 8 hours, compared with 3 hours for ARICEPT 10 mg tablets. Peak plasma concentrations were about 2-fold higher for ARICEPT 23 mg tablets than ARICEPT 10 mg tablets. ARICEPT ODT 5 mg and 10 mg are bioequivalent to ARICEPT 5 mg and 10 mg tablets, respectively. A food effect study has not been conducted with ARICEPT ODT; however, the effect of food with ARICEPT ODT is expected to be minimal. ARICEPT ODT can be taken without regard to meals. The elimination half life of donepezil is about 70 hours, and the mean apparent plasma clearance (Cl/F) is 0.13-0.19 L/hr/kg. Following multiple dose administration, donepezil accumulates in plasma by 4-7 fold, and steady state is reached within 15 days. The steady state volume of distribution is 12-16 L/kg. Donepezil 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. Donepezil 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. Donepezil is metabolized by CYP 450 isoenzymes 2D6 and 3A4 and undergoes glucuronidation. Following administration of ¹⁴C-labeled donepezil, plasma radioactivity, expressed as a percent of the administered dose, was present primarily as intact donepezil (53%) and as 6-O-desmethyl donepezil (11%), which has been reported to inhibit AChE to the same extent as donepezil in vitro and was found in plasma at concentrations equal to about 20% of donepezil. Approximately 57% and

15% of the total radioactivity was recovered in urine and feces, respectively, over a period of 10 days, while 28% remained unrecovered, with about 17% of the donepezil dose recovered in the urine as unchanged drug. Examination of the effect of CYP2D6 genotype in Alzheimer's patients showed differences in clearance values among CYP2D6 genotype subgroups. When compared to the extensive metabolizers, poor metabolizers had a 31.5% slower clearance and ultra-rapid metabolizers had a 24% faster clearance.

Absorption : Donepezil reaches peak plasma concentration in 3 to 4 hours and has a relative oral bioavailability of 100%. Plasma concentrations and the area under the curve rises proportionally according to dose and therefore the oral administration of donepezil produces highly predictable plasma concentrations. Administration of multiple single-daily doses results in a gradual approach to steady state as the terminal disposition half-life is approximately 70 hours. After initiation of therapy, approximate steady-state is achieved within 3 weeks. Over the course of the day at steady state, the related pharmacodynamic activity and plasma donepezil hydrochloride 2 concentrations show little variability. The absorption of donepezil hydrochloride is not affected either by time of administration (morning versus evening dose) or by food.

Distribution : Donepezil hydrochloride is approximately 96% bound to human plasma proteins. The steady state volume of distribution is 12L/kg. The donepezil distribution in various body tissues has not been studied definitively. In healthy male volunteers after a single 5mg dose of ¹⁴C - labeled donepezil hydrochloride in a mass balance study 240 hours after administration, approximately 28% of the label remained un-recovered. Expressed as a percentage of the concentration in plasma, the average CSF:plasma ratio for both doses, was 15.7%. Therefore donepezil and/or its metabolites may persist in the body for more than 10 day

Metabolism: Donepezil is extensively metabolised into four major metabolites, two of which are known to be active and a number of minor metabolites, not all of which have been identified. Donepezil's three human metabolites have not undergone extensive safety testing in animals. These metabolites comprise an N-oxidation product and two O-demethylated derivatives. Donepezil is metabolised by CYP450 isoenzymes, CYP3A4 and CYP2D6 and undergoes glucuronidation. Donepezil's rate of metabolism is slow and does not appear to be saturable. Formal pharmacokinetic studies are consistent with these

results which show that donepezil and/or its metabolites do not inhibit the metabolism of warfarin, theophylline, digoxin, or cimetidine in humans. Concurrent administration of cimetidine or digoxin in pharmacokinetic studies does not affect the metabolism of donepezil.

Excretion : Donepezil is excreted in the urine intact. Plasma radioactivity expressed as a percent of the administered dose, following administration of C14 - labeled donepezil, was present primarily as intact donepezil (53%), 6-O-desmethyl donepezil (11%), which has been reported to inhibit AChE to the same extent as donepezil in vitro and was found in the

plasma at concentrations equal to about 20% of donepezil. Over a period of 10 days, approximately 57% of the total radioactivity was recovered in urine and faeces, with about 17% of the donepezil dose recovered in the urine as unchanged, while 28% remained unrecovered. Donepezil plasma concentrations decline with a half-life of approximately 70 hours. There is no evidence to suggest enterohepatic recirculation of donepezil and/or any of its metabolites. Sex, smoking history and race have no clinically significant influence on plasma concentrations of donepezil.

Drug Interactions (36):

Table.4 Drug interactions of Donepezil Hydrochloride

DRUG	INTERACTIONS
<u>16-Bromoepiandrosterone</u>	The risk or severity of adverse effects can be increased when 16-Bromoepiandrosterone is combined with Donepezil.
<u>19-norandrostenedione</u>	The risk or severity of adverse effects can be increased when 19-norandrostenedione is combined with Donepezil.
<u>4-Androstenedione</u>	The risk or severity of adverse effects can be increased when 4-Androstenedione is combined with Donepezil.
<u>5-androstenedione</u>	The risk or severity of adverse effects can be increased when 5-androstenedione is combined with Donepezil.
<u>Abiraterone</u>	The serum concentration of Donepezil can be increased when it is combined with Abiraterone.
<u>Acebutolol</u>	Donepezil may increase the bradycardic activities of Acebutolol.
<u>Acetylcholine</u>	The risk or severity of adverse effects can be increased when Donepezil is combined with Acetylcholine.
<u>Aclidinium</u>	The therapeutic efficacy of Aclidinium can be decreased when used in combination with Donepezil.
<u>Alclometasone</u>	The risk or severity of adverse effects can be increased when Alclometasone is combined with Donepezil.
<u>Aldosterone</u>	The risk or severity of adverse effects can be increased when Aldosterone is combined with Donepezil.

MATERIALS AND METHODS:**Instruments used:****Table.5 List of instruments used**

S. No	Name of the Instrument	Make and model
1	HPLC	Agilent Technologies 1200 Infinity series
2	Electrical Balance	Metler Toledo ME204
3	Digital pH meter	EUTECH Instruments pH 700
4	Sonicator	PCI Analytics
5	Distillation Unit	Borosil
6	Vaccum Filtration Unit	Milli pore(XI O422050)

Chemicals used:**Table.6 List of chemicals used**

NAMES	CHEMICALS USED
Methanol	Merck Speciality Pvt .Ltd Mumbai
Water	Merck Speciality Pvt .Ltd Mumbai
Acetontrile	Merck Speciality Pvt .Ltd Mumbai

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS:**Selection of Solvent and Detection wavelength:**

The sensitivity of the HPLC method depends upon the proper selection of wavelength. Drug solution of 100µg/ml was scanned over the range of 200-400 nm in UV region using different solvents like methanol, hexane, ethanol, cyclohexane, Acetontrile and Water. It was observed that the drug showed maximum absorbance in Acetontrile and Water at 231 nm and hence Acetontrile and Water was used as solvents and 231nm was used as maximum wavelength for detection of Donepezil Hcl for further study.

Preparation of Standard Stock Solution:

Accurately weighed 100mg of Donepezil was dissolved in 100ml of HPLC grade Water and Acetontrile which was considered as a stock solution (1mg/ml). Working standard solutions were diluted further to get concentration range 10-50 µg/ml. Each of these drug solutions (10µl) were injected into the column, the peak area and retention time were recorded.

Preparation of Dilutions for Calibration Curve Construction:

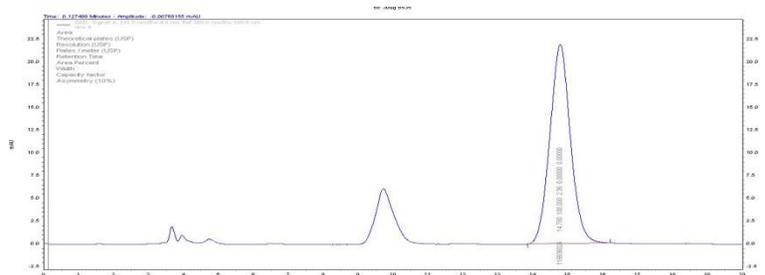
Dilute the working standard solution (100µg/ml) by pipetting 1,2,3,4 and 5ml of 100µg/ml solution into 10ml volumetric flasks and make up the volume with diluents Acetontrile:Water (50:50). This gives dilutions of 10, 20, 30, 40 and 50µg/ml solutions respectively.

Mobile Phase Selection:

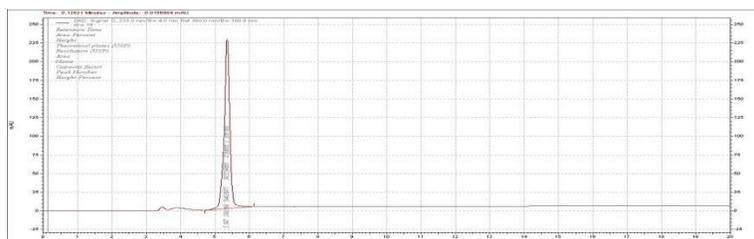
Experiments were conducted with mobile phase consisting of Acetontrile and Water. Trails were conducted taking different combinations of mobile phases to achieve maximum possible theoretical plates, least possible tailing factor and retention time. Based on this data, the best separation was obtained with 50:50 mobile phase composition.

RESULTS AND DISCUSSION:**HPLC METHOD DEVELOPMENT:**

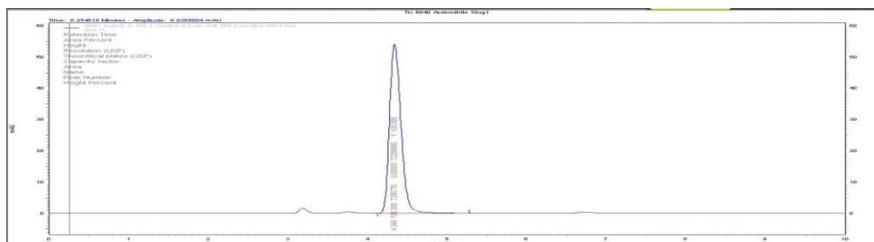
A reverse phase gradient liquid chromatographic technique was developed, optimised and validated for the determination of bulk form with UV detection at 231 nm by using unisol C18 column with mobile phase composition contains Acetontrile and Water in the ratio50:50 and in optimised isocratic program.

TRAIL: 1**Fig.10 Chromatogram of Trail 1**

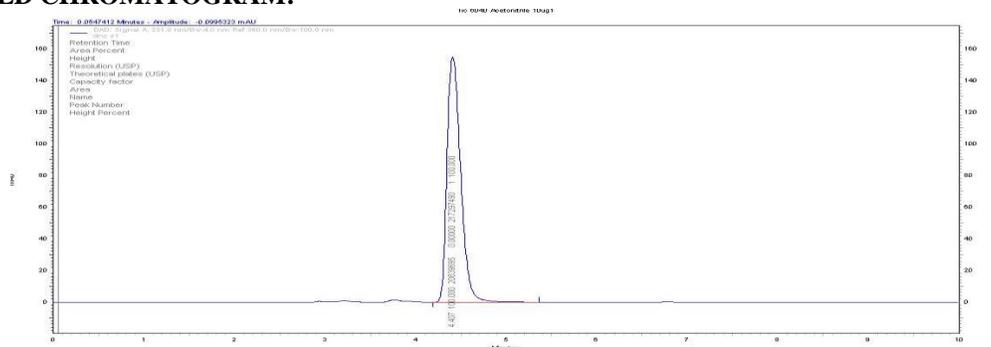
Discussion: Peak was eluted with moderate retention time but presence of high intensity peaks was observed even after many equilibrations. Base line distributions are observed, it was not satisfactory

TRAIL:2**Fig.11 Chromatogram of Trail 2**

Discussion: Peak was eluted with moderate retention time but presence of high intensity peaks was observed even after many equilibrations. base line distributions are observed, it was not satisfactory.

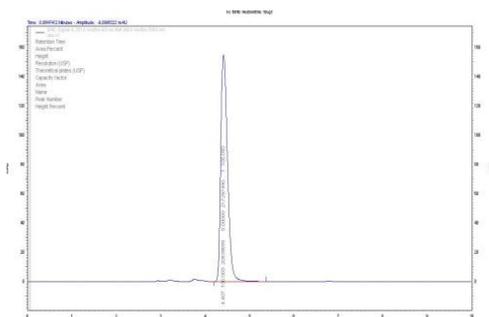
TRAIL:3**Fig.12 Chromatogram of Trail 3**

Discussion: When mobile phase composition was also used for the preparation of diluent, excellent results were observed. But base line disturbance was observed. flow rate was changed to make method more rapid and retention time was satisfactory.

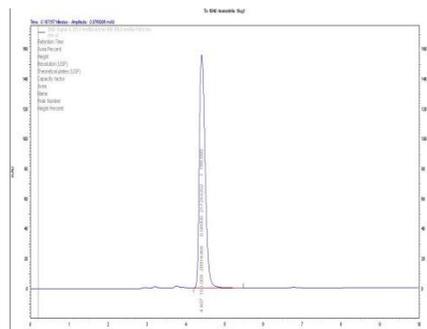
OPTIMIZED CHROMATOGRAM:**Fig.13 Optimized chromatogram**

Discussion: Symmetric peak with asymmetry factor not more than 2 and theoretical plates was obtained. Reproducible peak with excellent peak characteristics was obtained with rapid retention time of 4.40 minutes.

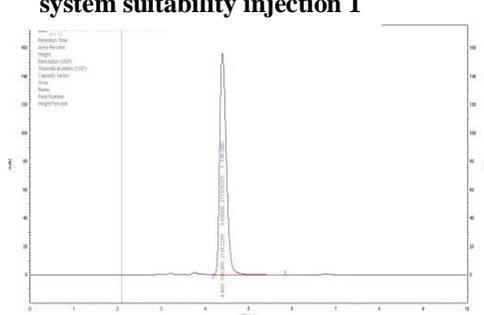
**METHOD VALIDATION:
SYSTEM SUITABILITY:**



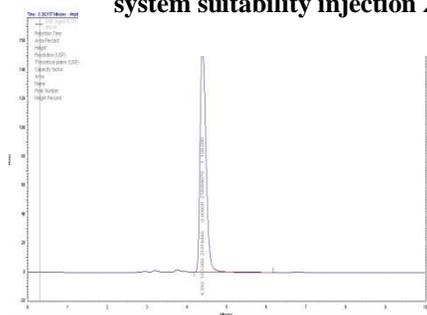
**Fig.14 Chromatogram for
system suitability injection 1**



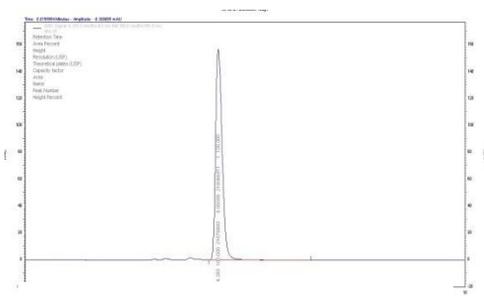
**Fig.15 Chromatogram for
system suitability injection 2**



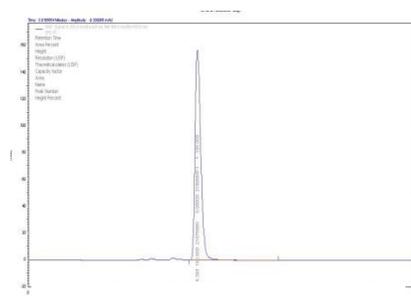
**Fig.16 Chromatogram for
system suitability injection 3**



**Fig.17 Chromatogram for
system suitability injection 4**



**Fig.18 Chromatogram for system
suitability injection 5**



**Fig.19 Chromatogram for
system suitability injection 6**

Table No.12 System suitability testing of Donepezil in HPLC

Injection number	Peak area	Theoretical plates	Tailing factor	Retention time
1	21039615	9749	1.05	4.40
2	21039618	9736	1.04	4.40
3	21039615	9723	1.03	4.40
4	21039613	9717	1.05	4.39
5	21039611	9708	1.05	4.39
6	21039611	9716	1.04	4.39
Mean	21039614	9724.8	1.043	4.35
%RSD	1.34	0.15	0.78	1.13
Acceptance criteria	NMT 2.0	NLT 2000	NMT 2.0	-

Inference: The obtained experimental values in system suitability trials (n=6) were found to be within the limits proposed by ICH guidelines.

SPECIFICITY:

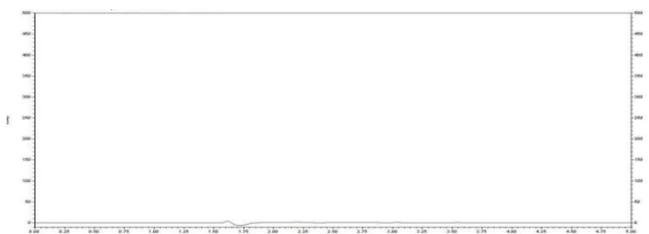


Fig.20 Blank chromatogram of Donepezil

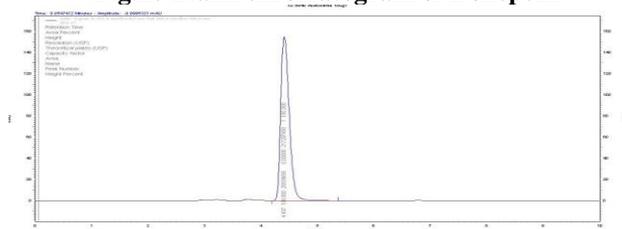


Fig.21 Standard chromatogram of Donepezil

Inference: Chromatograms of standard and blank were recorded and chromatogram of blank did not show any peak at the retention time of analyte. This shows that the method is specific.

LINEARITY AND RANGE:

The quantitative linearity was found in the concentration range of 10 to 50 µg/ml. The regression equation for linear range of concentrations was found to be $y = 54836x + 2E+06$.

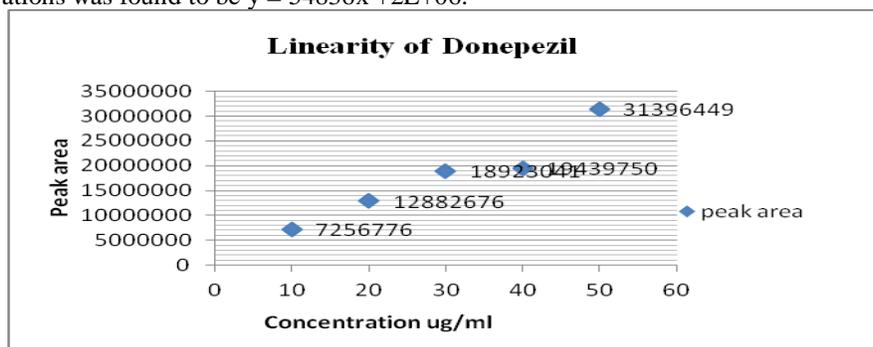


Fig.22 Calibration curve of Donepezil

Table No.13 Results for linearity in HPLC

Concentration(µg/ml)	Area
10	7256776
20	12882676
30	18923041
40	19439750
50	31396449
Correlation coefficient	0.999
Slope(m)	548364.2
Intercept(c)	1528812

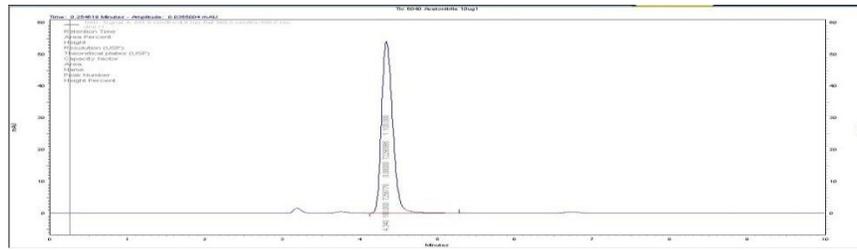


Fig.23 Linearity chromatogram of 10µg/ml of Donepezil

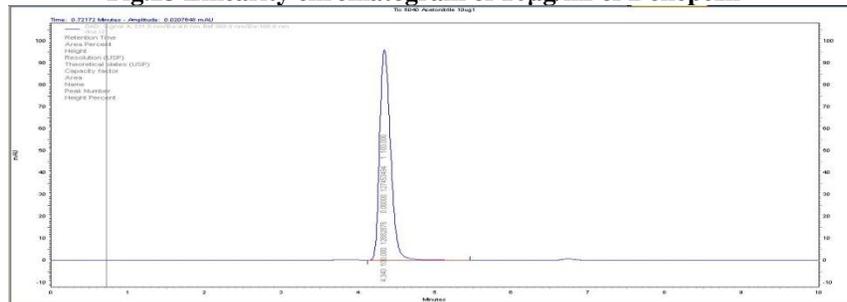


Fig.24 Linearity chromatogram of 20µg/ml of Donepezil

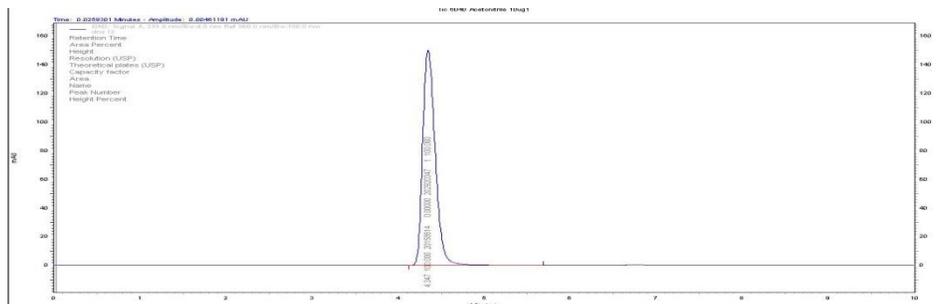


Fig.25 Linearity chromatogram of 30µg/ml of Donepezil

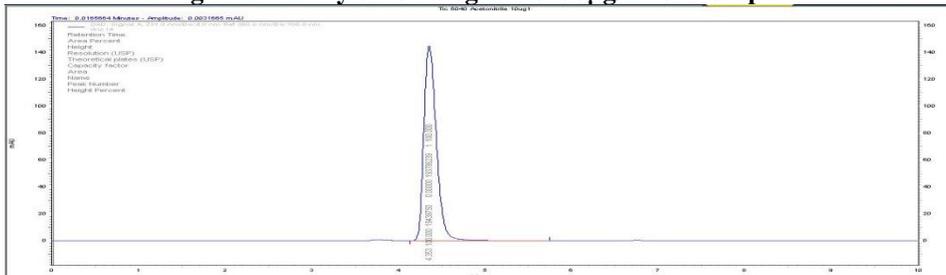


Fig.26 Linearity chromatogram of 40µg/ml of Donepezil

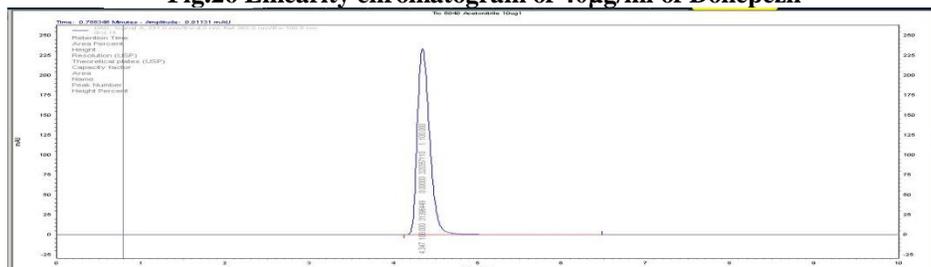
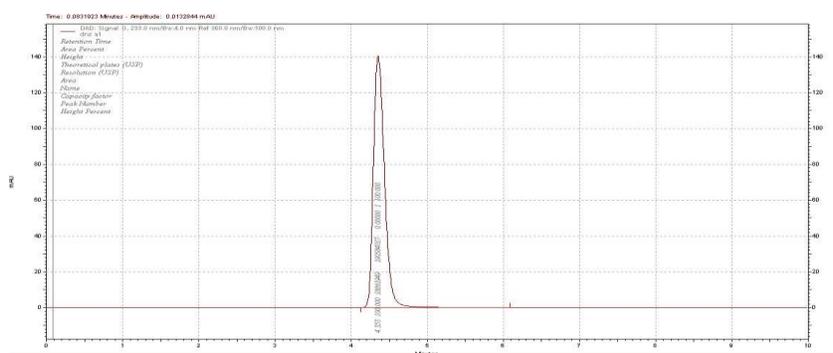
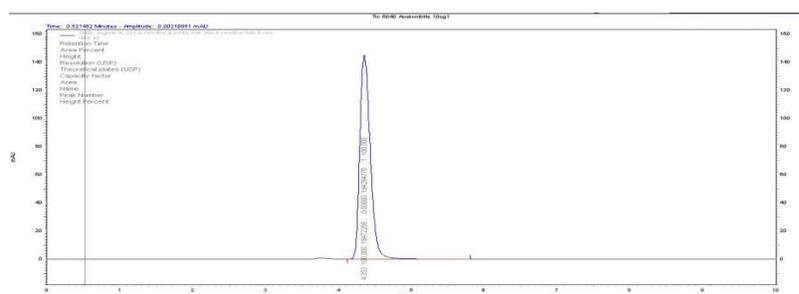
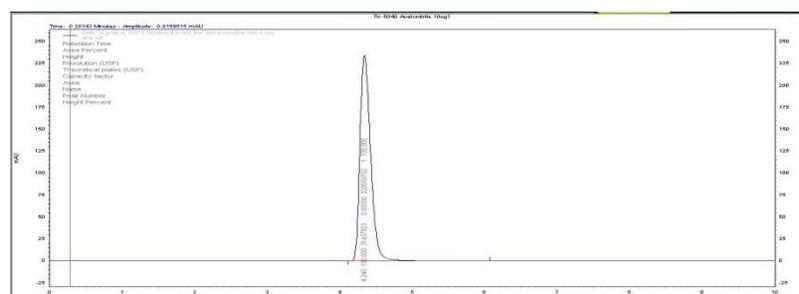


Fig.27 Linearity chromatogram of 50µg/ml of Donepezil

Inference: The response was found to be linear and the correlation coefficient was found to be 0.999.

ACCURACY:**Table No.14 Results for accuracy in HPLC**

S.NO	% Level	Standard amount	Spiked amount	Amount found	% Recovery	Mean Recovery
1.	50 %	20	10	29.81	99.68	Mean=99.26 SD = 0.5006 %RSD=0.50
		20	10	29.65	99.41	
		20	10	29.23	98.71	
2.	100 %	20	20	38.97	98.71	Mean =99. 20 SD = 0.436 %RSD=0. 44
		20	20	39.51	99.38	
		20	20	39.63	99.53	
3.	150 %	20	30	49.13	99.13	Mean =99. 03 SD =0. 087 %RSD=0. 09
		20	30	48.96	98.96	
		20	30	49.01	99.01	

**Fig.28 Accuracy chromatogram of 50% level concentraion****Fig.29 Accuracy chromatogram of 100% level concentraion****Fig.30 Accuracy chromatogram of 150% level concentration**

Inference: The results represent the high percent recovery values indicating that the proposed method is accurate.

PRECISION:

Interday Precision:

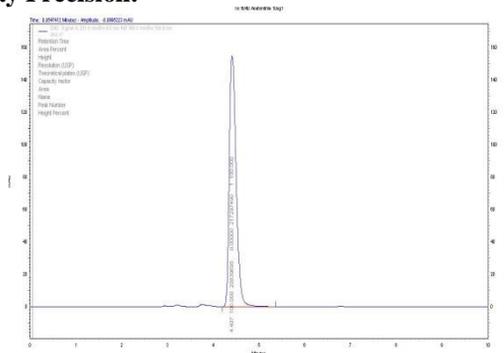


Fig.31 Chromatogram for intraday precision 1

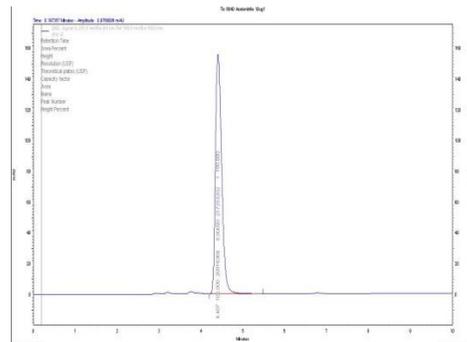


Fig.32 Chromatogram for intraday precision 2

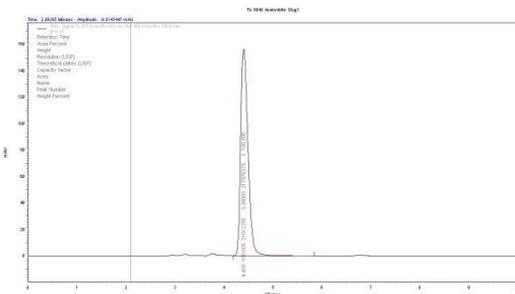


Fig.33 Chromatogram for intraday precision 3

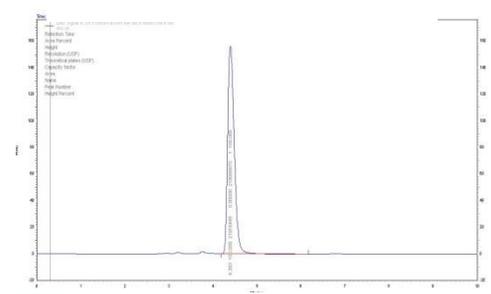


Fig.34 Chromatogram for intraday precision 4

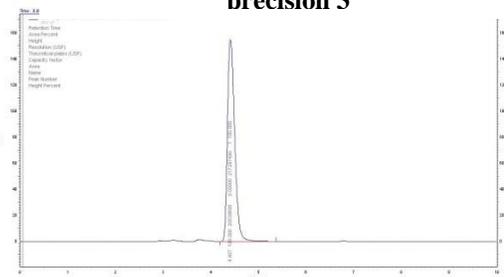


Fig.35 Chromatogram for intraday precision 5

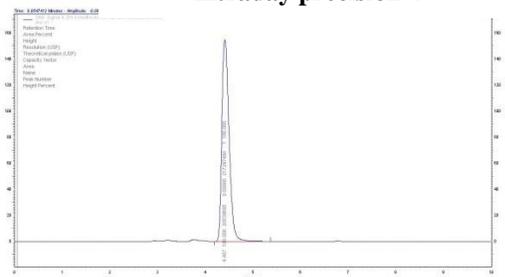


Fig.36 Chromatogram for intraday precision 6

Interday Precision:

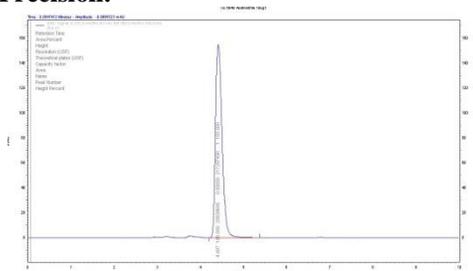


Fig.37 Chromatogram for interday precision 1

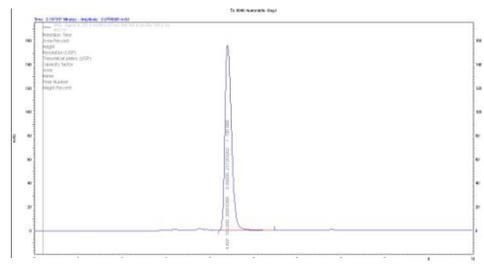


Fig.38 Chromatogram for interday precision 2

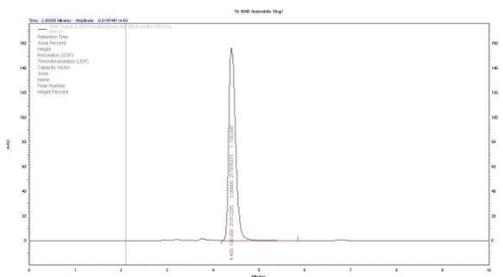


Fig.39 Chromatogram for interday precision 3

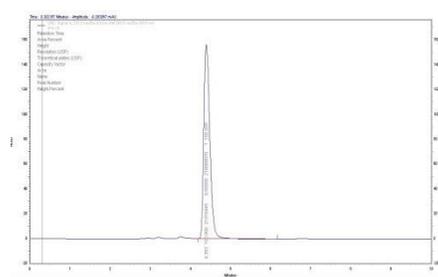


Fig.40 Chromatogram for interday precision 4

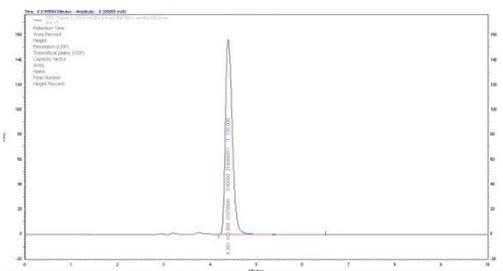


Fig.41 Chromatogram for interday precision 5

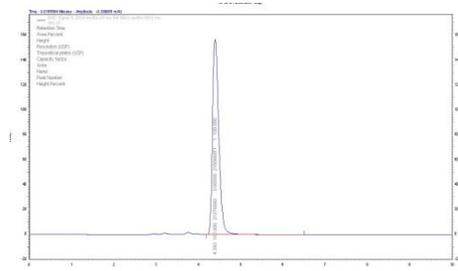


Fig.42 Chromatogram for interday precision 6

TABLE.13 RESULTS FOR Intraday and interday in HPLC

S.No	Intraday peak area	Interday peak area
1	21039615	21039510
2	21039618	21039528
3	21039615	21039569
4	21039613	21039592
5	21039611	2103587
6	21039611	21039553
%RSD	1.3	0.01

Inference: The % RSD for Intraday precision and interday precision for Donepezil were found to be 0.09 and 0.44 which indicates the method is precise.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD value was found at 1.8 µg/ml concentration where the signal to noise ratio was 3:1 and the LOQ value was found at 2.8 µg/ml where the signal to noise ratio was 10:1.

ROBUSTNESS:

Flow rate variation:

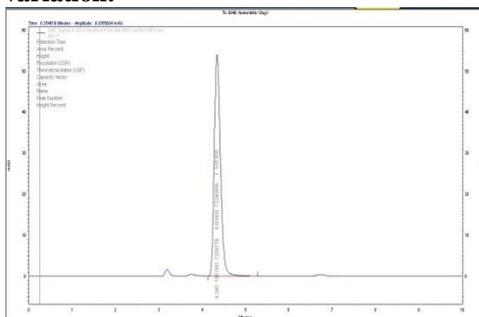


Fig.43 Chromatogram for Flow rate variation (0.8ml/min)

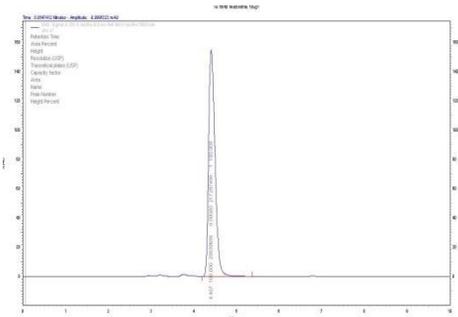


Fig.44 Chromatogram for Flow rate variation (0.4ml/min)

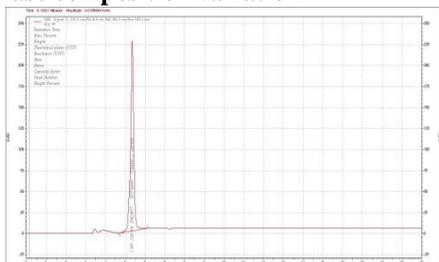
Mobile phase composition variation

Fig.45 Chromatogram for mobile phase composition variation (70:30)

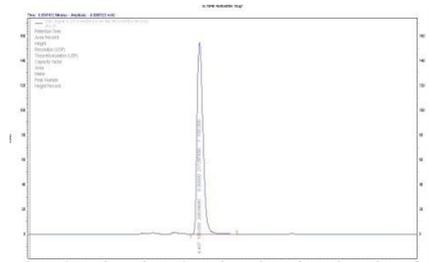


Fig.46 Chromatogram for mobile phase composition variation (50:50)

Table.16 Results for robustness in HPLC

Parameter	Conditions	%RSD (NMT 2.0)	Tailing factor (NMT 2.0)	Theoretical plates (NLT 2000)
Flow Rate	0.4	0.01	1.03	9981
	8.0	0.08	0.99	9147
	1.2	0.06	1.07	9291
Mobile phase composition	80:20	0.02	0.9	9513
	70:30	0.01	1.07	9712
	50:50	0.44	1.02	9314

Inference: All the experimental values for robustness obtained fall into the acceptance criteria.

SUMMARY AND CONCLUSION:**SUMMARY**

A simple, precise, economic, accurate, robust and LC-MS compatible Reverse phase High Performance Liquid Chromatographic method was developed for the analysis of Donepezil Hydrochloride. The initial trial was conducted with 10 minutes run time using Unisol reverse phase C18 column (150× 4.6 mm, 3µm) at room temperature conditions, 0.4ml/min flow rate and isocratic elution mode. The detection wavelength was fixed by scanning the working standard solution and noting the maximum absorbance wavelength which was found to be 231 nm and the mobile phase composed of Acetonitrile:Water (50:50).

The retention time was found at 4.407 mins. The calibration curve was linear with correlation coefficient of 0.999 over a concentration range of 10-50 µg/ml with linear regression equation $y = 54836x + 2E+06$. The limit of detection and limit of quantification were found at 1.8µg/ml and 2.8µg/ml respectively indicating the sensitivity of the method. Stability of the drug solution was checked for a period of 48 hrs and from the results it was found that the drug solution is stable without undergoing degradation. The proposed method has been validated according to the ICH guidelines and can be successfully applied to estimate the levels of Donepezil in bulk form.

Table.23 Summary for RP-HPLC method

S.No	Parameter	Acceptance criteria	Results obtained
1	System suitability	Theoretical Plates-NLT 2000	9724.8
2	Specificity	No interference of blank	Passed
3	Linearity	Correlation coefficient NLT 0.996	0.998
4	Precision Intra Day Inter Day	%RSD NMT 2	1.3
		%RSD NMT 2	0.01
5	Accuracy	Percentage Recovery 98-102%	99.68

REFERENCES:

- Shethi P. Quantitative Analysis of Pharmaceutical Formulations. 2011.
- United States Pharmacopoeia and National Formulary, (24th) Asian Edition, The United States Pharmacopoeia Convention Inc. U.S.A. 2126.
- Sethi PD, HPLC: Quantitative Analysis of Pharmaceutical Formulation, New Delhi, CBS Publishers and Distributors, 1996; 113-202.
- sankar S. Text book of Pharmaceutical Analysis. 5th ed. 2006,12-13.
- Jeffery GH, Bassett J, Mendham J, Denny RC, Vogel's Textbook of Quantitative Chemical Analysis, fifth edition, Longman scientific & technical.
- Kaushal C, Srivastava B, A Process of Method Development: A Chromatographic Approach. J Chem Pharm Res, 2010; 2(2): 519-545.
- Buffers and pH Buffers: available from: www.xtremepapers.com. Accessed April 05, 2013.
- Understanding pH Buffers: which one to use, and at what concentration: available from: www.laserchrom.co.uk. Accessed April 05, 2013.
- Technical Tips: Selecting Buffers pH in Reversed-phase HPLC: available from: 0020download.5117.com/data/file/30.pdf. Accessed April 05, 2013.
- Reversed-phase HPLC Buffers: High Quality Buffers (solutions, solids or concentrates): available from: ccc.chem.pitt.edu/wipf/web/HPLC_RP_buffers.pdf. Accessed April 05, 2013.
- Effective HPLC method development. Available from http://www.cvg.ca/images/HPLC_Method_Development.pdf. Accessed April 05, 2013.
- Changhe Wen, Designing HPLC Methods for Stability Indication and Forced Degradation Samples For API, Collected from American; Pharmaceutical Review at <http://www.americanpharmaceuticalreview.com>. Accessed April 05, 2013.
- Chandra M., Buffers: A guide for the preparation and use of buffers in biological system: available from: www.calbiochem.com. Accessed April 05, 2013.
- Lindholm J, Development and Validation of HPLC Method for Analytical and Preparative Purpose, Acta Universities Upsaliensis Uppsala, 2004; 13-14.
- Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC method development. 2nd edition. New York. John wiley.1997; 233-291.
- <http://dx.doi.org/10.1002/9781118592014>
- Columns. From http://www.waters.com/watersdivision/pdf/Ic3A_C.pdf. Accessed April 05, 2013.
- Wagaw S, Tedrow J, Grieme T, Bavda L, Wang W, Viswanath S et al. HPLC Guide; Departments R450, R452, R45R.
- Weston A, Brown PR, HPLC and CE Principles and Practise, Academic press, California, 1997.
- Donald D. Hong and Mumtaz Shah, Development and validation of HPLC Stability-indicating Assays, In: Sens T. Carstensen, C.T.Rhodes, editors Drug Stability-Principle & Practice. 3rd Edition. New York: Marcel Dekker Inc. 2008; p. 332.
- John W. Dolan, "Stability -Indicating Assays", LC Troubleshooting 2005, 275.
- Dean JA, Analytical Chemistry Handbook, Mc Graw-Hill, New York, 1995.
- Mayer ML. HPLC for Pharmaceutical scientist. Am. Lab. 1997; 29, 34-37.
- U.S. FDA, Title 21 of the U.S. Code of Federal Regulations: 21 CFR 211- Current good manufacturing practice for finished pharmaceuticals.
- U.S. FDA - Guidance for Industry (draft) Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls and Documentation, (2000).
- ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories, (2005).
- International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: definitions and terminology, Q2A, Geneva (1996).
- International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: Methodology, Q2B, Geneva (1996).
- U.S. EPA, Guidance for methods development and methods validation for the Resource Conservation and Recovery Act (RCRA) Program, Washington, D.C. (1995), <http://www.epa.gov/sw-846/pdfs/methdev.pdf>
- General Chapter 1225, Validation of compendial methods, United States Pharmacopeia 30, National Formulary 25, Rockville, Md., USA, The United States Pharmacopeial Convention, Inc., (2007).
- Horacio Pappa, Romina Farru', Paula Otan'õ Vilanova, Marcelo Palacios, Mari'a Teresa Pizzorno *A new HPLC method to determine

- Donepezil hydrochloride in tablets.
31. Stability Assessment Of Donepezil Hydrochloride Using Validated RP-HPLC Method Paresh P Chothe*, Ramaa C Subramanian, Vilasrao J Kadam
 32. Analytical method development and validation of donepezil hydrochloridetablets by rphplc.t. senthil kumar1*, p. solairaj2, a.thangathirupathi3
 33. Stability indicating analytical method development and validation of memantine hcl and donepezil hcl using rp-hplc
 34. Syeda noorain amena*1, s. h. rizwan2. analytical method developement and validation for the
 35. Simultaneous estimation of memantine hcl and donepezil hcl in bulk and pharmaceutical dosage form rajgor vm1, parmar pt2, patel cn3, patel as1