MATERIALS AND METHODS
PROCUREMENT AND MAINTENANCE OF MICE

Male albino mice, *Mus musculus* of 30 days age, (20±2 g) were selected as experimental animals. The mice were purchased from Indian Institute of Science (IISc), Bangalore, animals were held in separate polypropylene cages with *ad libitum* access to feed and water in an air-conditioned environment (25±2 °C) with a 12-h light and 12-h dark cycle. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (vide No.:05/(i)/a/ CPCSCA/ IAEC/ SVU/ KY/BNK/Dt. 22.09.2007).

DRUG SELECTED

Galantamine hydrobromide was selected for the present study. It was obtained from Sigma Aldrich (Apollo Pharmaceuticals, Hyderabad).

PHYSICAL AND CHEMICAL PROPERTIES OF GALANTAMINE HYDROBROMIDE

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Galantamine hydrobromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brand Name</td>
<td>Razadyne</td>
</tr>
<tr>
<td>Discovered by</td>
<td>former Soviet Union scientists, Janssen</td>
</tr>
<tr>
<td>Discovered in</td>
<td>1950s in Bulgaria</td>
</tr>
<tr>
<td>Drug Category</td>
<td>Nootropic Agents; Parasympathomimetics</td>
</tr>
<tr>
<td>Physical State</td>
<td>White crystalline powder</td>
</tr>
<tr>
<td>Organisms Affected</td>
<td>Humans and other mammals</td>
</tr>
<tr>
<td>Route</td>
<td>Oral</td>
</tr>
<tr>
<td>Compound ID</td>
<td>121587</td>
</tr>
<tr>
<td>CAS Number</td>
<td>1953-04-04</td>
</tr>
<tr>
<td>Pubchem</td>
<td>9651</td>
</tr>
<tr>
<td>ATC Code</td>
<td>N06DA04</td>
</tr>
<tr>
<td>Drug Bank</td>
<td>APRD00206</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C17H22BrNO3, C17H21NO3-HBr</td>
</tr>
</tbody>
</table>
Molecular Weight | 368.26548 [g/mol]
--- | ---
Chemical IUPAC Name | (4aS,6R,8aS)-4a,5,9,10,11,12-Hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2-e][2]benzazepin-6-ol
--- | ---
Chemical Structure | ![Chemical Structure Image]
--- | ---
Melting Point | 262-270°C
--- | ---
Bioavailability | 80 to 100%
--- | ---
Protein binding | 18%
--- | ---
Half life | 7 hours
--- | ---
Metabolism | Hepatic partially CYP450:CYP2D6/3A4 substrate
--- | ---
Excretion | Renal (95%, of which 32% unchanged), fecal (5%)
--- | ---

**AGE OF MICE CORRESPONDING TO HUMAN BEINGS**

<table>
<thead>
<tr>
<th>Age of mice</th>
<th>Corresponding age of human being</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>30 months (2.5 years)</td>
</tr>
<tr>
<td>2 months</td>
<td>60 months (5 years)</td>
</tr>
<tr>
<td>3 months</td>
<td>90 months (7.5 years)</td>
</tr>
<tr>
<td>4 months</td>
<td>120 months (10 years)</td>
</tr>
<tr>
<td>5 months</td>
<td>150 months (12.5 years)</td>
</tr>
<tr>
<td>6 months</td>
<td>180 months (15 years)</td>
</tr>
</tbody>
</table>

**SELECTION OF THE SOLVENT**

In the present study, 0.9% of normal saline used as a solvent to dissolve the test chemical, galantamine hydrobromide.
**Table 1:** Schedule of Experiment for analysis of morphometric and Behavioural aspects and biochemical assays in control and experimental mice treated with Galantamine hydrobromide.

**Experimental Protocol:**

<table>
<thead>
<tr>
<th>Name of the Batch</th>
<th>Age of mice</th>
<th>Duration of treatment</th>
<th>Time schedule for Biochemical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chronic</td>
<td></td>
</tr>
<tr>
<td>Batch I Control</td>
<td>30 days</td>
<td>15d 30d 45d 60d 75d 90d 105d 120d 135d 150d 165d 180d 45d 60d 75d 90d 105d 120d 135d 150d 165d 180d 210d 240d</td>
<td></td>
</tr>
<tr>
<td>Batch II Experimental</td>
<td>30 days</td>
<td>15d 30d 45d 60d 75d 90d 105d 120d 135d 150d 165d 180d 45d 60d 75d 90d 105d 120d 135d 150d 165d 180d 210d 240d</td>
<td></td>
</tr>
</tbody>
</table>
ROUTE OF ADMINISTRATION OF THE DRUG

After the mice were acclimated to the laboratory conditions, they were divided into several groups depending on the dosage and time for sacrificing the animals. Twenty four groups of six each were housed in separate cages. The time periods chosen for experiments following administration of the drug were 15 to 180 days (Table. 1). All doses were given in the morning between 7.30 to 8.30 am, keeping in view the altered activity of mice during the nights compared to day time. Controls were maintained individually for each group. The dose for oral administration of Galantamine, according to ED₅₀ value (the dosage that produces a desired effect in half of test population) obtained in mice was 5mg/kg body wt (Sweeney et al., 1990).

EVALUATION OF THE EFFECTIVE DOSE

The Reed-Muench method (1938) is a simple method for determining the ED₅₀ value in experimental biology. The ED₅₀ was checked by maintaining ten groups, six mice per group up to one month. In that, five groups were control and another five groups were experimental treated with different doses (1mg, 3mg, 5mg, 10mg and 15mg) of galantamine hydrobromide for one month orally with the help of gavage. The drug effect was observed on the morphometric and behavioural aspects for every five days. From this data, the effective dose of galantamine hydrobromide to mice was determined as 5mg/kg body weight.

The following experiments have been conducted on selected days namely 15th, 30th, 45th, 60th, 75th, 90th, 105th, 120th, 135th, 150th, 165th and 180th day of exposure of mice to Galantamine hydrobromide against the control mice.

MORPHOMETRIC STUDIES

The basic morphometric aspects such as the size and total body weight of the control and experimental mice treated with different doses of galantamine hydrobromide have been recorded once in five days up to 180 days. The data thus obtained was analysed and used to correlate with the behavioural and biochemical aspects.

BEHAVIOURAL ASPECTS

The changes in the behaviour pattern of the experimental mice treated with chronic doses of galantamine hydrobromide will be recorded with help of the Morris water maze technique.
Prior to the experiment the animals were acclimatize to maze environment. For the present study, the animals were divided into 24 batches each batch consisting of 6 animals. Among them, 12 batches were labeled as control and remaining 12 batches as experimental. The water maze experiment was conducted for both control and experimental animals on the selected days viz. 15th, 30th, 45th, 60th, 75th, 90th, 105th, 120th, 135th, 150th, 165th and 180th for all six animals in a group separately and the time taken to reach the hidden platform was noted down and average was calculated. Improvement in learning and memory activity in mice were determined by comparing the time for between the control and the experimental mice to assess the performance skills and also the impact of galantamine hydrobromide on the overall behavioural pattern of mice.

**Morris water maze**: The water maze was originally designed to test the ability of animals to learn and memorize the location of a hidden platform in a pool of opaque water by its position relative to distal extra maze cues (Morris et al., 1982). This learning task was selected because a great deal of knowledge has been obtained on the neurochemical, neuroanatomical and neurophysiological basis for the behaviours associated with this paradigm (Mc Namara and Skelton, 1993). The water maze is a circular tank measuring 1.85m in diameter and 0.7m deep. Constructed according to a basic design similar to that of Morris (Morris, 1984). The pool was filled to a depth of 30cm with milky water and kept at 22-25oC. A circular submerged platform (Diameter 12.5cm) below the surface of water was placed in position. All parameters involving time and distance were measured in seconds.

**BIOCHEMICAL ASSAYS**

**ISOLATION OF TISSUES**

The control and experimental mice were sacrificed as per the experimental schedule by cervical dislocation. Brain was isolated immediately and placed on a chilled glass plate. The brain areas viz. Olfactory Lobe, Hippocampus, Cerebral Cortex, Cerebellum, Ponsmedulla and Spinal Cord were isolated by following standard anatomical marks (Glowinski and Iversen, 1966). These are separated and frozen in liquid nitrogen (-180°C) and stored at -40°C until future studies.

38
METHODS FOR ESTIMATIONS OF NEUROTRANSMITTERS AND ENZYMES

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the Parameter</th>
<th>Methods employed</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CHOLINERGIC SYSTEM</td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>Acetylcholine</td>
<td>Augustinsson (1957)</td>
</tr>
<tr>
<td>b)</td>
<td>Acetylcholsterase</td>
<td>Ellmann et al., (1961)</td>
</tr>
<tr>
<td>II</td>
<td>GLUTAMATE METABOLISM</td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>Glutamine Content (GC)</td>
<td>Colowick and Kaplan (1967)</td>
</tr>
<tr>
<td>b)</td>
<td>Glutamine Synthetase</td>
<td>Wu et al., (1963)</td>
</tr>
<tr>
<td>c)</td>
<td>Glutamate dehydrogenase (GDH)</td>
<td>Lee and Lardy (1965)</td>
</tr>
<tr>
<td>d)</td>
<td>Glutaminase activity (GA)</td>
<td>Alton and Meister (1955)</td>
</tr>
</tbody>
</table>

I. CHOLINERGIC SYSTEM

a) Acetylcholine (ACh): Acetylcholine (ACh) content was estimated by the method of Metcalf (1957) as given by Augustinsson (1957). Different areas of the brain like Olfactory Lobe, Hippocampus, Cerebral Cortex, Cerebellum, Ponsmedulla and Spinal Cord were weighed accurately, transferred to test tubes and placed in a boiling water bath for 5 minutes to terminate the Acetylcholinesterase enzyme activity and also to release the bound Acetylcholine (ACh). Then the tissues were homogenized in 1 ml of distilled water. To the homogenate, 1 ml of alkaline hydroxylamine hydrochloride was added followed by 1 ml of 50% hydrochloric acid solution. The contents were mixed thoroughly and centrifuged. To the supernatant, 0.5 ml of 0.37 M ferric chloride solution was added and the brown colour developed was read at 540 nm against a reagent blank (1 ml of distilled water and 0.5 ml of 0.37 M ferric chloride solution) in a spectrophotometer. The acetylcholine content was expressed as µ moles of ACh/g wet weight of tissue.

b) Acetylcholinesterase (AChE): (E.C.: 3.1.1.7; Acetylcholine acetyl hydrolase): Acetylcholinesterase (AChE) activity was estimated by the method of Ellman et al., (1961). 10% homogenates of different brain areas were prepared in 0.25 M ice-cold sucrose solution. The reaction was started with the addition of 100 µ liters of homogenate to the reaction mixture containing 3.0 ml of phosphate buffer (pH 8.0),
20μ moles of substrate, Acetylcholine iodide (0.075M) and 100μ moles of Dithiobis trinitrobenzoic acid (DTNB 0.01M). The developed colour was read at 412 nm in a spectrophotometer. The enzyme activity was expressed as μ moles of ACh hydrolyzed/mg protein/hour.

II. GLUTAMATE METABOLISM

a) Glutamine content (GC) : Glutamine content was estimated by acid hydrolysis method as described by Colowick and Kaplan (1967). 10% homogenates of the tissue were prepared in cold distilled water and the homogenates were centrifuged at 1000 rpm for 15 minutes. To 0.1 ml of the supernatant, 0.2 ml of 10% H₂SO₄ was added and the tubes were kept in boiling water-bath for 15 minutes and cooled. The contents were centrifuged and to the supernatant, 0.3 ml of 10% NaOH was added and the mixture was made up to 2.0 ml with distilled water. Ammonia was estimated by nesslerization. The glutamine content was expressed as μ moles of ammonia/gm wet weight of the tissue.

b) Glutamine synthetase (GS) (L-glutamate ammonia ligase) (E.C.: 6.3.1.2) : The activity of Glutamine synthetase was estimated by the method of Wu (1963) modified by Ramamurthy et al., (1982). Tissue homogenates (10%) were prepared in distilled water and used as the enzyme source. The reaction mixture in a volume of 1 ml contained 0.4 ml imidazole HCl buffer (pH 7.2), 0.1 ml MgCl₂ (0.2M), 0.1 ml 2-mercaptopethanol, 0.1 ml glutamate (0.5M, pH 7.2), 0.1 ml ATP (0.1M), 0.1 ml hydroxylamine (1M, pH 7.2), and 0.1 ml of enzyme. The reaction mixture was incubated at 37°C for 15 minutes and 1.5 ml of ferric chloride solution was added to the tubes and the contents were centrifuged. The clear supernatants were read at 535 nm against a reagent blank in a UV/VIS spectrophotometer (Hitachi, Model U-2000, Japan). The enzyme activity was expressed as μ moles of glutamyl hydroxamate formed/mg tissue/hr.

c) Glutamate dehydrogenase (GDH) (L-Glutamate NAD oxido-reductase) (E.C.: 1.4.1.3) : The activity of GDH was assayed by the method of Lee and Lardy (1965). Tissue homogenate (10%) was prepared in ice-cold 0.25M sucrose solution and centrifuged at 1000 rpm for 15 minutes. The supernatant was used as the enzyme source. The reaction mixture in a volume of 2 ml contained 100 μ moles of phosphate buffer (pH 7.2), 4.0 μ moles of sodium glutamate, 0.1 μ moles of NAD, 4 μ moles of
INT and 0.1 ml of enzyme source. The reaction mixture was incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 5.0 ml of glacial acetic acid. The formazan formed was extracted overnight at 5°C into 5.0 ml of rectified toluene. The intensity of colour developed was read against toluene at 495 nm in a UV/VIS Spectrophotometer (Hitachi, Model U-2000, Japan). The Glutamate dehydrogenase activity was expressed as μ moles of formazan/mg protein/hr.

d) Glutaminase activity (GA) (L-Glutamine amino hydrolase) (E.C.: 3.5.1.2): Glutaminase activity was estimated by the method of Alton and Meister (1955). 5% homogenates of the tissues were prepared in ice-cold distilled water and centrifuged at 2000 rpm for 15 min. The supernatant was used as the enzyme source. The reaction mixture in a total volume of 1 ml contained 50 μ moles of L-glutamine prepared fresh in sodium acetate buffer (pH 4.9) and 0.5 ml of the enzyme source. The contents were incubated at 37°C for 30 minutes and the reaction was stopped by adding 1 ml of 10% TCA. The contents were centrifuged and to the supernatant 1 ml of 15% NaOH and 1 ml of nessler’s reagent were added. The intensity of the colour developed was read at 490 nm against the blank in a Spectrophotometer. The enzyme activity was expressed in μ moles of Ammonia released/mg protein/hr.

VALIDITY OF EXPERIMENTAL PROCEDURES

1. General: For all the enzyme studies in the present investigation, the assays were standardized by conducting preliminary assay to determine the optimal pH, temperature, enzyme and substrate concentration and these optimal conditions were subsequently followed for each enzyme assay.

2. Aliquots for Assay: Aliquots selected for the assay were such that the initial rates were approximately as near as possible, yet providing sufficient product of fall in a convenient range for Spectrophotometric measurement.

3. Enzyme units: The soluble protein content of tissue homogenate (enzyme source) was estimated using Folin phenol reagent (Lowry et al., 1951). This was used for the expression of enzyme activity. Enzyme activities were expressed in standard units i.e., μ moles of product formed or substrate cleaved/mg protein/h or g wet weight of the tissue/h.
4. **Substrate requirements**: All the enzyme activity levels were determined at saturating substrate concentrations i.e., in zero order.

5. **Beer-Lambert law**: Almost all the products of the reactions were measured by using the colorimetric procedures in which the optical density of the resulting coloured complexes was proportional to the concentration of reaction products.


**Statistical treatment of the data**: All assays were carried out with six separate replicates from each group. All values are expressed as Standard Error Mean (SEM) and test of significance or students 't' test. Difference between control and experimental assays was considered as significant at P<0.05 (Pillai and Sinha, 1968). Standard deviation and probability test i.e., 't' test were employed to know the levels of significance.

**Standard deviation was calculated with the following formula**

\[ SD = \bar{x} - \frac{(\bar{x}/n)2}{n-1} \]

Where, \( x \) = individual observations

\( n \) = total number of observations

**'t' test was calculated by using the following formula**

\[ t = \frac{x_1 - x_2}{SD_1^2 + SD_2^2} \sqrt{(n_1 + n_2) - 2} \]

Where, \( x_1 \) = mean of the control

\( x_2 \) = mean of the experimental

\( SD_1 \) = Standard deviation of the control

\( SD_2 \) = Standard deviation of the experimental

\( n_1 \) = Number of observations of the control

\( n_2 \) = Number of observations of the experimental

**Percent Deviation** = \( \frac{Cm - Em}{Cm} \times 100 \)

Where \( Cm \) = Mean of the control value

\( Em \) = Mean of the experimental value
APPLICATION OF LIPINSKI'S RULE OF FIVE TO ANTI ALZHEIMER'S DRUGS AND EVALUATION OF THEIR TOXIC PROPERTIES

Another main aim of the present work is "validation of Lipinski rule of five for some selected anti Alzheimer's drugs" such as Galantamine hydrobromide (GHBr), Physostigmine, Rivastigmine, Eptastigmine, Memantine, Xanomeline, Huperzine A and E2020 (Aricept™) which are currently recommended to treat Alzheimer's disease. Validation of chemical drugs was done by:

a) Random selection of different drugs used to treat Alzheimer's disease.

b) Obtaining SMILES format for these drugs using software's.

c) Applying 'Lipinski's rule of five' for each of these drugs.

Finally, resolving violation of the Lipinski rule.

Software's used

Molinspiration tool: Molecular properties and bioactivity of the drugs showing high affinity predicted using Molinspiration server. This server allows physico chemical properties to calculate Log P based on group contributions. The values were obtained by fitting calculated logP with experimental logP. PSA is good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and Blood brain barrier penetration.

Molinspiration: Molinspiration is an independent research organization focused on development and application of modern Cheminformatics techniques, especially in connection with the internetmolinspiration offers broad range of Cheminformatics software tools supporting molecule manipulation and processing, including SMILES and SDfile conversion, normalization of molecules, generation of tautomers, molecule fragmentation, calculation of various molecular properties needed in QSAR, molecular modeling and drug design, high quality molecule depiction, molecular database tools supporting substructure search or similarity and pharmacophore similarity search. Our products support also fragment-based virtual screening, bioactivity prediction and data visualization. Molinspiration tools are written in Java, therefore are available practically on any computer platform.

Molinspiration supports also internet chemistry community by offering free on-line cheminformatics services for calculation of important molecular properties (for example logP, polar surface area, number of hydrogen bond donors and
acceptors), as well as prediction of bioactivity score for the most important drug targets.

SMILES C1/C1=CC3=C(C1(C=C2=C1)N(C)C)C=CC(=O)N3

The above picture shows all the components of the Lipinski’s rule i.e.

- miLogP — A partition coefficient log P
- MW — Molecular Weight
- nON — Not more than 10 hydrogen bond acceptors
- nOHNH — Not more than 5 hydrogen bond donors

It also gives a plan of:

- natsoms — number of heavy atoms
- TPSA — Total Polar Surface Area
- nrotb — number of rotatable bonds
- nviolations — number of violations made by the proposed drug (nvio-used
 later)
- volum — the volume of the compound (vol. — used later)

The software also gives the design of the canonical smiles of the same chemical being used.

LogP (octanol/water partition coefficient): It is used in QSAR studies and rational drug design as a measure of molecular hydrophobicity. Hydrophobicity affects drug absorption, bioavailability, hydrophobic drug-receptor interactions, metabolism of molecules, as well as their toxicity. LogP has become also a key parameter in studies
of the environmental fate of chemicals. LogP is calculated by the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors. Method is very robust and is able to process practically all organic and most organometallic molecules.

**Total Molecular Polar Surface Area (TPSA)**: It is a very useful parameter for prediction of drug transport properties. Polar surface area is defined as a sum of surfaces of polar atoms (usually oxygens, nitrogens and attached hydrogens) in a molecule. This parameter has been shown to correlate very well with the human intestinal absorption, Caco-2 monolayers permeability, and blood-brain barrier penetration. It is calculated based on the methodology published by Ertl *et al.*, 2000 as a sum of fragment contributions. O- and N- centered polar fragments are considered. PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood-brain barrier penetration.

**Molecular Volume (MV)**: Molecular volume determines transport characteristics of molecules, such as intestinal absorption or blood-brain barrier penetration. Volume is therefore often used in QSAR studies to model molecular properties and biological activity. Various methods may be used to calculate molecular volume, including methods requiring generation of 3D molecular geometries, or fragment contribution methods such as McGowan volume approximation. Method for calculation of molecule volume developed at Molinspiration is based on group contributions. These have been obtained by fitting sum of fragment contributions to "real" 3D volume for a training set of about twelve thousand, mostly drug-like molecules. 3D molecular geometries for a training set were fully optimized by the semiempirical AM1 method.

"Rule of 5" Properties: It is set of simple molecular descriptors used by Lipinski in formulating his "Rule of 5" (Lipinski *et al.*, 1997). The rule states, that most "drug-like" molecules have logP <= 5, molecular weight <= 500, number of hydrogen bond acceptors <= 10, and number of hydrogen bond donors <= 5. Molecules violating more than one of these rules may have problems with bioavailability. The rule is called "Rule of 5", because the border values are 5, 500, 2*5, and 5.

**Number of Rotatable Bonds - rotb**: This simple topological parameter is a measure of molecular flexibility. It has been shown to be a very good descriptor of oral bioavailability of drugs (Veber *et al.*, 2002). Rotatable bond is defined as any
single non-ring bond, bounded to nonterminal heavy (i.e., non-hydrogen) atom. Amide C-N bonds are not considered because of their high rotational energy barrier.

**OSIRIS Software**

The OSIRIS Property Explorer is a online Chemoinformatics tool. It used to predict Toxicity Risk Assessment, cLogP value, Molecular Weights, Solubility, Drug-Likeness Prediction and Overall Drug-Likeness Score of the drug molecule. It lets you draw chemical structures and calculates on-the-fly various drug-relevant properties whenever a structure is valid. Prediction results are valued and color coded. Properties with high risks of undesired effects like mutagenicity or a poor intestinal absorption are shown in red. Whereas a green color indicates drug-conform behaviour. Drug likeliness was also calculated using OSIRIS server, which is based on a list of about 5,300 distinct substructure fragments created by 3,300 traded drugs as well as 15,000 commercially available chemicals yielding a complete list of all available fragments with associated druglikeliness. The drug score combines drug-likeliness, cLogP, logS, molecular weight, and toxicity risks as a total value which may be used to judge the compound's overall potential to qualify for a drug.

**Molsoft software**

Molsoft is a California based software company that is a primary source of new breakthrough technologies in: Molecular graphics and visualization, Molecular modeling, Docking and Virtual screening, computational biology and Cheminformatics. All molecular property predictors are calculated using fragment-based contributions. It developed an original method for splitting a molecule into a set of linear or non-linear fragments of different length and representation levels and counting the number of occurrences of each chemical pattern found. A Partial Least Squares (PLS) regression model was built and optimized for a particular property using a leave-50%-out cross-validation calculation. The method is very robust and fast (about 5K of compounds per second).